Molecular and Physiological Characterization of the Flowering Time Control Protein, HvFCA and its Role in ABA Signalling and Seed Germination

BY

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TABLE OF CONTENTS

LIST OF TABLE(S)

LIST OF FIGURES

ABBREVIATIONS

ABA	Abscisic Acid
ABAP1	ABA Binding Protein 1
ABI5	Abscisic Acid Insensitive 5
FCA	Flowering Time Control Protein A
FLC	Flowering Control Locus C
FT	Flowering Locus T
GA	Gibberellic Acid
GFP	Green Fluorescent Protein
GUS	Beta-D-glucuronidase
LEA	Late Embryogenesis Abundant
LUC	Luciferase
ORF	Open Reading Frame
PHS	Pre-harvest Sprouting
RFP	Red Fluorescent Protein
RRM	RNA Recognition Motif
siRNA	Small Interfering RNA
SLN1	Slender 1 (DELLA protein)
VP1	Viviparous 1
WW	Protein Interaction Domain
Em	Early Methionine

ABSTRACT

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The RNA binding protein Flowering Time Control Locus A (FCA) regulates flowering in rice and Arabidopsis. The abscisic acid binding protein ABAP1 shares high sequence homology to FCA and was considered the FCA homologue in barley. The current study investigates the existence of *ABAP1* as an independent gene product and also the cloning, characterization and functional significance of the gamma (γ) isoform of *FCA* from barley.

Barley FCA protein showed higher sequence similarity to wheat and rice FCA compared to Arabidopsis FCA. It contains two RNA recognition motifs (RRMs), a glycine rich region at the N-terminal end, the WW domain and a poly-glutamine region immediately downstream of WW domain at the C-terminal. In developing barley embryos, *FCA* transcripts could be detected from 2 days after pollination (DAP) up to late maturity without any detectable change within these stages. *FCA* transcript levels declined as germination progressed in barley embryos and the *FCA* transcripts were retained for longer duration when germination was reduced with application of ABA. *FCA* also showed up-regulation by ABA and abiotic stresses in barley germinating seeds and seedlings. Transient

co-expression of barley *FCA* or a truncated *FCA* (lacking RRM) with a maize *VP1* promoter-GUS construct or a wheat *Em* gene promoter-GUS construct in barley aleurone layer protoplasts led to increased GUS activity in both cases. Adding ABA during the incubation enhanced the observed increase due to *FCA* expression. Similar effects of transient over-expression of *FCA* in barley embryos affected *VP1*. Barley FCA localized to the nucleus. This nuclear localization was due to the nuclear localization signal within the protein and not due to the RNA recognition motifs (RRMs) as the truncated FCA lacking RRMs also localized to the nucleus. Barley *FCA* did not restore the flowering phenotype in an Arabidopsis *fca-1* mutant. In conclusion, I have shown that barley *FCA* is up-regulated by ABA and stress in embryos and affects ABA signalling in barley caryopses. The properties of FCA appear to have diverged between dicot and monocot systems.

1.0 INTRODUCTION

The plant hormone abscisic acid (ABA) affects a variety of physiological processes including seed dormancy, stomatal conductance and various biotic and abiotic stresses (Finkelstein *et al.* 2002, Ton *et al.* 2009, Wasilewska *et al.* 2008). Many ABA signalling intermediates have been identified and characterized with respect to their various functions but ABA receptors remained elusive until recently when a number of ABA receptors were identified using genetic and biochemical approaches. The first two ABA-binding proteins, ABAR and ABAP1 were identified using biochemical approaches in broad bean and barley respectively (Razem *et al.* 2004, Zhang *et al.* 2002). The Arabidopsis homologues of ABAR and ABAP1 were identified based on sequence similarity to these two proteins and were characterized as ABA receptors (Razem *et al.* 2006, Shen *et al.* 2006).

The current study was undertaken to understand the role of *ABAP1* in barley and to find its relationship to barley *FCA*. ABAP1 was isolated from a barley aleurone layer expression library using an antibody that recognized the ABA binding sites on the protein and was characterized as an ABA binding protein, but its physiological function was unknown (Razem *et al.* 2004). The two important characteristics of ABAP1, ABA binding and its exclusive presence and up-regulation by ABA in seed tissue suggested its role in seed germination. The Arabidopsis counterpart of ABAP1, FCA an RNA binding protein, was known to regulate flowering (Macknight *et al.* 1997) but FCA had not been identified in barley. ABAP1 shares high sequence similarity to FCA at the C-terminal region

and the WW domain required for protein-protein interaction is conserved between the two proteins. Although the ABA binding region is common to both the proteins, ABAP1 lacks the RNA recognition motifs (Razem *et al.* 2004, Razem *et al.* 2006). It has also been speculated that *ABAP1* is probably a truncated fragment of *FCA* (Finkelstein 2006). However, since the *FCA* gene is known to undergo alternative splicing, the possibility of *ABAP1* being an alternatively-spliced variant of *FCA* gene could not be ruled out.

It has subsequently been shown, independently in two laboratories, that there were significant errors in the data related to ABA binding by FCA resulting in the simultaneous publication of a note describing the problem (Risk *et al.* 2008) and a retraction by the authors of the paper (Razem *et al.* 2008). The results describing ABA binding by ABAP1 (Razem *et al.* 2004) were also shown to be non-reproducible and a retraction of that manuscript is forthcoming.

Recent progress in understanding the mechanism of functioning of FCA has shed some light on various molecular events that control FCA function. In Arabidopsis, FCA was shown to interact with various small interfering RNA and proteins that control chromatin modification and epigenetic regulation of physiological events (Baurle *et al.* 2007, Sarnowski *et al.* 2002). The interaction of FCA with FY, a 3'-polyadenylation factor, which was thought to be strong and stable, is now in fact considered transient leading to altered organization of splicing machinery on FY (Manzano *et al.* 2009). This finding is critical when there is more than one report about FCA being involved in generation or processing of signals from the surrounding environment to activate cellular responses (Furner *et*

al. 1996, Manzano *et al.* 2009). The role of FCA in flowering is partially conserved in (dicots) Arabidopsis and (monocot) rice despite differences in mechanism of control of flowering, suggesting the existence of multiple targets for FCA (Lee *et al.* 2005). The interaction of FCA and FY has mainly been implicated in floral transition but the possibility of the existence of additional targets other than floral transition has not been discounted (Marquardt *et al.* 2006). Various reviews have pointed toward a common mechanism regulating flowering and dormancy (Horvath 2009). The expression of the *FCA* homologue in poplar is up-regulated when apical dormancy is triggered in the tree, suggesting an alternative role for FCA (Ruttink *et al.* 2007).

The current study was undertaken to gain insights into the role of barley FCA and ABAP1 with the following objectives: (i) Tracing the origin/existence of *ABAP1* and its similarity/distinctiveness from barley *FCA*. (ii) Cloning and characterization of barley *FCA* with regards to dormancy and germination in barley. (iii) Molecular and physiological function of barley FCA in ABA signalling with regards to dormancy**.**

2.0 LITERATURE REVIEW

2.1 Introduction to barley

2.1.1 History of barley

Barley (*Hordeum vulgare* L.) is one of the oldest crops of ancient world agriculture. Archaeological remains of barley grains were found at various sites in the Fertile Crescent (Zohary and Hopf 1993) indicating that the crop was domesticated about 8,000 BC. The wild relatives of barley were known as *Hordeum spontaneum* C. Koch. In modern taxonomy, *H. vulgare* L. and *H. spontaneum* C. Koch, as well as *Hordeum agriocrithon* Åberg, are considered subspecies of *H. vulgare* (Bothmer and Jacobsen 1985). Studies with molecular markers have confirmed that barley was brought into cultivation in the Isreal-Jordan area but barley diversification occurred in Indo-Himalayan regions (Badr *et al.* 2000).

2.1.2 Importance of barley in Canada

Barley, a gladiator's food in Athens and the only crop to be used as a form of money in early Sumerian and Babylonian cultures, is the fourth largest cultivated crop in the world after wheat, rice and maize. Barley is one of the most fundamental plants in human nutrition and it is one of the most highly adapted of the cereal grains grown in climates ranging from sub-Arctic to subtropical (Zohary and Hopf 1993). Barley is classified as either six-row or two-row, depending on the physical arrangement of the kernels on the plant. Barley can be described as

hulled or hull-less depending upon the presence or absence of covering on the kernels respectively.

In Canada, barley was first grown in Port Royal in 1606. Today, Canada is the fourth largest producer of barley after the European Union, Russia and Ukraine (Taylor *et al.* 2009). Most farmers grow barley for sale as malting barley. If the grain does not meet malting quality, it is sold as feed barley. Malting quality is somewhat subjective and depends upon the supply of good malting barley and its price. In the past couple of years, barley crops have suffered great loss in yield and quality due to lower germination potential and water sensitivity (Statistics Canada, 2007). Despite significant losses in barley production and yield in the year 2006-2007 (9.5 million tonnes (Mt)), the total production of barley increased (11.8 Mt) in 2007-2008 due to larger cropping area at the expense of wheat acerage (Statistics Canada, 2007).

Total barley production decreased by 10% and the harvested area by 1.5% in 2009 compared to 2008. Domestic use has increased by 4% due to a decline in corn imports. Total exports have increased by 12.5% in 2009 after a drastic decline of 47% in 2008 from the previous year (USDA Report, 2009). Average price for malt barley has gone down significantly from $208 to $179 per tonne (Agric. & Agri-Food Canada, 2009).

2.1.3 Challenges related to barley production in Canada

Malting quality characteristics (beta-glucan content, protein breakdown, fermentability, hull adherence and even germination) are extremely important

aspects for barley improvement. While considerable progress has been achieved, much remains to be done in terms of improving the quality and production of malting barley. The problems associated with germination of Canadian malting barley have gained considerable attention over the past few years, both domestically and internationally. While it is clear that pre-harvest sprouting and storage conditions have a significant effect on germination behaviour, the underlying causes for varietal differences in these characteristics is still unclear. Secondary dormancy greatly reduces the germination and marketability of grains used for malting purposes. The challenge is to ensure that malting barley maintains its germination, but without pre-harvest sprouting or prolonged dormancy.

2.2 Seed dormancy

2.2.1 Definition of seed dormancy

Seed dormancy is a very common trait of wild plants that ensures their survival under unfavourable conditions, decreases competition with other species and prevents damage to seedlings from out-of-season germination of the seed. Domesticated species, on the other hand, are selected for uniform germination and rapid seedling establishment often leading to selection of genotypes with less dormancy. This can lead to pre-harvest sprouting (PHS), a phenomenon in which the seed germinates on the parent plant causing extensive loss of grain quality to crops like wheat, barley and maize (Bewley and Black 1994).

Seed dormancy is defined as inhibition of germination of an intact viable seed under favourable conditions (Bewley 1997, Hilhorst 1995, Li and Foley 1997). This block to germination has evolved differently in different plant species depending upon their habitat and conditions of growth. In keeping with the variety of climates and habits in which these germination blocks operate, diverse dormancy mechanisms have evolved. In light of the complex nature of germination blocks, a more sophisticated and experimentally useful definition of dormancy is stated as, a “dormant seed cannot germinate in a specified period of time under any combination of conditions that are otherwise sufficient for its germination” (Baskin and Baskin 2004). Dormancy should not be associated with absence of germination, rather it is the combination of the characteristics of the seed that determines the physical and environmental conditions required for its germination (Finch-Savage and Leubner-Metzger 2006). Germination can be defined as emergence of the radicle from the seed coat. The requirement for germination may include one or more of the processes like mobilization of stored food, overcoming the physical barrier by activation of cell wall degrading enzymes followed by resumption of active growth by cell elongation and division (Finkelstein *et al.* 2008).

2.2.2 Classification of seed dormancy

Although almost all kinds of dormancy cause delay in germination, the underlying causes may vary. This variation can be due to embryonic immaturity or due to physiological and physical constraints caused by the presence of a hard

seed coat or some inhibitory chemicals that interfere with embryo growth (Finch-Savage and Leubner-Metzger 2006). Dormancy can be a primary dormancy that is acquired during the later stages of embryo development and seed maturation. There are also conditions in which after-ripened, imbibed seeds enter into secondary dormancy when exposed to unfavourable temperature, light or low moisture conditions (Bewley 1997).

Despite the progress in understanding the mechanisms controlling dormancy, it is described as one of the least understood phenomena (Finch-Savage and Leubner-Metzger 2006). Both physiologists and ecologists have studied the factors controlling dormancy but the outcome is far from clear due to the fact that dormancy is affected by numerous environmental conditions (an ecologist's dilemma) and the model species like Arabidopsis studied by molecular physiologists and geneticists tend to have a very shallow dormancy (Gubler *et al.* 2005, Walck *et al.* 2005). The molecular controls that regulate dormancy have either an embryo component or a seed coat component; however, dormancy itself is a whole seed characteristic and on that basis, is classified into the five classes namely physiological, morphological, morpho-physiological, physical and combinatorial dormancy (Baskin and Baskin 2004, Finch-Savage and Leubner-Metzger 2006, Nikolaeva 1969).

2.2.3 Factors affecting dormancy

Dormancy is affected by various factors and the potential regulators are identified by their effect on depth of dormancy or by analysis of genetic lines that

have varying levels of dormancy. The factors that affect dormancy are classified into two broad categories, seed coat imposed and embryo imposed dormancy. A hard seed coat manifests its effect on dormancy by prevention of water uptake during imbibition (waxy or lignified tissues in legume seeds), mechanical constraint due to hard seed coat (nuts) or endosperm (lettuce) causing inhibition of radicle protrusion, interference with gas exchange (cocklebur) and retention of inhibitors (*Xanthium*) and production of inhibitors like ABA. Genetic variation in seed coat components such as testa layer, pericarp and pigmentation also cause altered dormancy and seed longevity (Debeaujon *et al.* 2000, Groos *et al.* 2002, Sweeney *et al.* 2006). Pigmented seeds are generally more dormant although hormone levels and their sensitivity to them may increase dormancy of non-pigmented seeds (Flintham 2000, Gale *et al.* 2002, Walker-Simmons 1987). Many nitrogenous compounds like NO gas, nitrite (NO_2^-) and nitrate (NO_3^-) cause dormancy release. In Arabidopsis, NO may stimulate germination by causing vacuolation and cell wall weakening (Bethke *et al.* 2007). Genomic studies in rice to identify loci controlling seed colour, dormancy and shattering resistance show a tight linkage between the responsible genes. Some of these traits may be controlled by a single locus (Ji *et al.* 2006).

Embryo dormancy is controlled by inherent characteristics of the embryo. The presence or absence of embryo dormancy has mainly been attributed to the content and sensitivity of phytohormones abscisic acid (ABA) and gibberellic acid (GA) (Bewley 1997). Dormancy and germination are also affected by environmental factors such as moisture, temperature and light (Borthwick *et al.*

1952, Gutterman *et al.* 1996). The setting of dormancy during seed development on the mother plant, and its intensity in mature seeds varies greatly according to the genotype and the environmental conditions throughout seed development (Bewley 1997, Corbineau *et al.* 2000, Crome *et al.* 1984).

2.2.4 Hormonal control of dormancy

The plant hormone abscisic acid is required for setting dormancy during embryo maturation and its accumulation correlates with the onset of primary dormancy (Kermode 2005). Another plant hormone, gibberellic acid is antagonistic in action to ABA. Gibberellins promote post-germinative growth by activating hydrolyzing enzymes that break cell walls, mobilize seed storage reserves and stimulate embryo cell expansion (Bewley 1997). Ethylene also promotes germination by antagonizing ABA signalling. Ethylene receptor mutants have higher ABA content and are hypersensitive to ABA (Beaudoin *et al.* 2000, Chiwocha *et al.* 2005, Ghassemian *et al.* 2000). Plant steroidal hormones, brassinosteroids, enhance the germination potential of embryos in a GA-independent manner (Leubner-Metzger 2001). Auxin is involved in germination completion and seedling establishment (Carrera *et al.* 2007, Liu *et al.* 2007b, Ogawa *et al.* 2003). Auxin accumulation occurs during embryo development at the radicle tip before germination and in seeds after imbibition (Liu *et al.* 2007b). Although various hormones may affect dormancy and germination, the general consensus is that ABA is the primary mediator of dormancy (Finkelstein *et al.* 2008, Holdsworth *et al.* 2008a, Koornneef *et al.* 2002).

2.2.5 Cross talk between ABA and GA in regulation of dormancy and germination

The role of ABA in induction and maintenance of dormancy is firmly established and widely reviewed (Finch-Savage and Leubner-Metzger 2006, Finkelstein *et al.* 2008, Koornneef *et al.* 2002, Nambara and Marion-Poll 2003). In cereals such as barley, wheat and *Sorghum*, development of dormancy also appears to be under ABA control (Jacobsen *et al.* 2002, Walker-Simmons 1987). Genetic studies show that *de novo* synthesis of ABA in embryo or endosperm is required to induce dormancy (Nambara and Marion-Poll 2003). Other studies with ABA-deficient mutants have suggested that ABA in the embryos and not the maternal ABA is important for induction of dormancy (Karssen *et al.* 1983). Dormancy may be maintained by renewed post-imbibition synthesis of ABA (Ali-Rachedi *et al.* 2004, LePage-Degivry and Garello 1992). Dormancy is reduced in seeds that are mutants for ABA biosynthetic enzymes, that have ABA sequestration with expressed antibodies in the seeds and in seeds that are treated with chemicals for inhibition of ABA biosynthesis (Lin *et al.* 2007, Nambara and Marion-Poll 2003). ABA content and the resulting dormancy are controlled by interaction of ABA biosynthetic and ABA catalyzing enzymes. The most critical enzyme in ABA biosynthesis is the 9-*cis*-epoxycarotenoid dioxygenase (NCED) that is essential for ABA synthesis in embryo and endosperm (Lefebvre *et al.* 2006). The rate-limiting enzyme in ABA biosynthesis, NCED regulates ABA biosynthesis during induction of secondary dormancy

(Leymarie *et al.* 2008). During the transition from embryo maturation to germination, ABA is catabolised by ABA 8'-hydroxylases encoded by the cytochrome P450 CYP707A family of genes causing a decline in dormancy (Okamoto *et al.* 2006). Imbibition of embryos in water also causes leaching of ABA resulting in reduced dormancy (Suzuki *et al.* 2000). After-ripening, which is occurring during dry storage of seeds, causes a decline in embryo ABA content and sensitivity (Ali-Rachedi *et al.* 2004, Grappin *et al.* 2000). In a study conducted on pre-harvest sprouting (PHS) susceptible and resistant wheat cultivars, after-ripening occured before harvest ripeness in the most PHS-susceptible cultivars, whereas it was slowest in the most PHS-resistant cultivars. However, no direct relationship could be found between timing of caryopsis after-ripening and dormancy or ABA responsiveness in wheat (Gerjets *et al.* 2009).

Not only ABA content but also ABA sensitivity are critical components of embryo dormancy. ABA-insensitive mutants that are deficient in ABA perception or signalling have lower dormancy and exhibit viviparous germination (Koornneef *et al.* 1984, Koornneef *et al.* 1989, Robichaud and Sussex 1986). Analysis of sprouting-susceptible and sprouting-resistant cultivars of wheat for ABA content and ABA sensitivity showed larger differences in ABA sensitivity than ABA content measured by capability of ABA to block embryo germination (Walker-Simmons 1987).

The role of GA in modulating dormancy is highly debated (Finkelstein *et al.* 2008). GA treatment alone does not lead to germination in all species or in fully-dormant Arabidopsis seeds. A decline in ABA is usually required before embryo

GA content or sensitivity to the hormone increases (Ali-Rachedi *et al.* 2004, Jacobsen *et al.* 2002). After-ripening, which leads to a decline in ABA content and ABA sensitivity, results in increased sensitivity to GA and light in Arabidopsis (Derkx and Karssen 1993). So the ratio of ABA to GA seems to be critical, where a higher content of ABA overrides the growth-promoting effect of GA. In cereals, although the GA signalling components seem to be similar to dicots, redundant GA signalling pathways may exist. This is evident from the fact that mutation in the only known GA receptor, *Gibberellin-Insensitive Dwarf 1* (*GID1*) in rice leads to decreased α-amylase production (Ueguchi-Tanaka *et al.* 2005); however mutating all the three homologues of *GID1* in Arabidopsis inhibits germination (Willige *et al.* 2007). This suggests that the embryo dormancy in cereals, for the most part, is controlled by ABA content and sensitivity.

2.2.6 Effect of light on dormancy occurs through ABA and GA metabolism

The role of light in regulation of dormancy was first identified when irradiation of dark-imbibed seeds with a red (R) light pulse induced germination and a subsequent far-red (FR) light pulse cancelled the effect of R light (Borthwick *et al.* 1952). This response is mediated by the R/FR light photoreceptor phytochromes, UV-A/blue light receptor cryptochromes, the blue light receptor phototropins and the recently identified blue light receptor zeitlupes (Bae and Choi 2008, Seo *et al.* 2009).

The induction of germination by red light can be substituted by the application of GA (Kahn *et al.* 1957), whereas the application of red light cannot

induce germination in GA-deficient mutants (Oh *et al.* 2006). The expression of GA biosynthetic genes encoding GA3ox (*AtGA3ox1* and *AtGA3ox2* in Arabidopsis and *LsGA3ox1* in lettuce) is induced by R light and the induction is inhibited by FR light (Toyomasu *et al.* 1998, Yamaguchi *et al.* 1998). In contrast, transcript levels of a GA-deactivating gene *GA2ox* (*AtGA2ox2* in Arabidopsis and *LsGA2ox2* in lettuce) are decreased by R light treatment (Nakaminami *et al.* 2003, Oh *et al.* 2006, Seo *et al.* 2006, Yamauchi *et al.* 2007).

Similar to modulation of GA content, ABA biosynthetic and deactivating enzymes are also regulated by light. ABA biosynthetic genes encoding zeaxanthin epoxidase (AtZEP/AtABA1 in Arabidopsis) and NCED (*AtNCED6* and *AtNCED9* in Arabidopsis and *LsNCED2* and *LsNCED4* in lettuce) are decreased by R light treatment (Oh *et al.* 2007, Sawada *et al.* 2008, Seo *et al.* 2006). In contrast, transcript levels of ABA-deactivating genes encoding CYP707A (CYP707A2 in Arabidopsis and *LsABA8ox4* in lettuce) are elevated by R light treatment (Oh *et al.* 2007, Sawada *et al.* 2008, Seo *et al.* 2006).

The phytochromes regulate the levels of ABA and GA by one of their interacting proteins *PHYTOCHROME INTERACTING FACTOR3-LIKE 5* (*PIL5*) which belongs to a family of helix-loop-helix (bHLH) class of proteins containing 15 members (Toledo-Ortiz *et al.* 2003, Yamashino *et al.* 2003). Studies of *PIL5* over-expressing and mutant lines show that it regulates ABA and GA content by regulating their metabolic genes (Oh *et al.* 2006).

2.3 Molecular Networks regulating dormancy

2.3.1 Perception and transduction of ABA signal

2.3.1.i ABA Receptors

Physiological experiments with numerous plant species have indicated that accumulation of ABA is required for dormancy induction and maintenance (Finkelstein *et al.* 2008). The perception of ABA and subsequent downstream signalling to activate ABA-regulated responses is an area of active research. Various lines of evidence suggested that there are multiple sites of ABA perception, thus, multiple ABA receptors (Allan *et al.* 1994, Gilroy and Jones 1994, Huang *et al.* 2007). The first ABA-specific binding protein, a 42 kDa ABAR, was isolated from broad bean (*Vicia faba*) leaves and the pretreatment of their guard cell protoplasts with a monoclonal antibody raised against the 42-kD protein significantly decreased the ABA specific-induced phospholipase D activity in a dose-dependent manner (Zhang *et al.* 2002). Another 52kDa protein, ABAP1 was shown to bind ABA and was up-regulated by ABA in barley aleurone layer tissue (Razem *et al.* 2004). An ABA "receptor", the Flowering Time Control Locus A (FCA) in Arabidopsis was identified based on its high sequence similarity to barley ABAP1 and was shown to bind ABA and affect flowering (Razem *et al.* 2006). Another ABA receptor from Arabidopsis, the Magnesium Protoporphyrin-IX Chelatase H subunit (CHLH) regulates classical ABA-regulated processes like stomatal movements, seed germination and post germination growth (Shen *et al.* 2006). CHLH also shared very high sequence similarity to ABAR (Shen *et al.*

2006). In 2008, questions about FCA being a receptor for ABA arose in both the laboratory of the original authors and, independently, in laboratories in New Zealand and Japan. This culminated in the simultaneous publication of a letter questioning the original results (Risk *et al.* 2008) and a retraction of the claim that FCA was an ABA receptor (Razem *et al.* 2006). Subsequent studies have confirmed that the results in the original Razem *et al.* (2006) paper were not reproducible (Jang *et al.* 2008, Risk *et al.* 2009). Questions have also been raised regarding CHLH and its effect on feedback regulation of ABA synthesis and the apparent lack of a mechanism for its function as an ABA receptor (Shen *et al.* 2006, Verslues and Zhu 2007). CHLH binding to ABA was proven using more than one method (Wu *et al.* 2009). Yet the barley homologue of CHLC (magnesium chelatase 150 kD subunit) does not bind ABA (Muller and Hansson 2009). In recent years, two classes of plasmamembrane ABA receptor, a G-protein-coupled receptor (GPCR), GCR2, and a novel class of GPCR, GTG1 and GTG2, that regulate the major ABA responses in seed germination, seedling growth, and stomatal movement have been discovered (Liu *et al.* 2007c, Pandey *et al.* 2009). However, the GCR2 regulation of ABA-mediated seed germination and post-germination growth are controversial because the ABA-related phenotypes are weak to absent in *gcr2* mutants (Gao *et al.* 2007, Guo *et al.* 2008). GTGs are positive regulators of ABA signalling and interact with the sole Arabidopsis G-protein α-subunit, GPA1, that may negatively regulate ABA signalling by inhibiting the activity of GTG-ABA binding (Pandey *et al.* 2009). The ABA insensitive mutants *abi1* and *abi2* belong to Mg^{2+}- and Mn^{2+}-dependent

serine-threonine phosphatases type 2C (PP2Cs) and are known to be negative regulators of ABA signalling (Gosti *et al.* 1999, Merlot *et al.* 2001, Meyer *et al.* 1994, Rodriguez *et al.* 1998). The 14 member gene family of Regulatory Components of ABA Receptor (RCARs), which interact with ABI1 and ABI2, were shown to bind ABA, mediate ABA-dependent inactivation of ABI1 and ABI2 *in vitro* and antagonize PP2C action *in planta* (Ma *et al.* 2009). PYRABACTIN RESISTANCE 1 (PYR/PYL family of START proteins) were shown to inhibit the PP2C mediated ABA signaling (Park 2009). The PYR/PYL/RCAR family members were classified as the major *in vivo* phosphatase 2C-interacting proteins in Arabidopsis (Noriyuki *et al.* 2009). The crystal structure of Arabidopsis PYR1 indicated that the molecule existed as a dimer, and the mechanism of its binding to ABA in one of the PYR1 subunits was recently established (Nishimura *et al.* 2009, Santiago *et al.* 2009). Finally, the whole ABA signalling cascade that includes PYR1, PP2C, the serine/threonine protein kinase SnRK2.6/OST1 and the transcription factor ABF2/AREB1 was reconstituted *in vitro* in plant protoplasts resulting in ABA responsive gene expression (Fujii *et al.* 2009).

2.3.1.ii ABA signalling components

To identify the different ABA signalling components, various Arabidopsis mutants were screened for insensitivity to ABA for germination and were termed ABA insensitive (*abi*) (Finkelstein 1994, Koornneef *et al.* 1984). The ABI1 and ABI2 encoded protein phosphatase 2C (type 2C phosphatases, PP2C) are involved in ABA signalling (Leung *et al.* 1997, Meyer *et al.* 1994). *ABI3*, *ABI4* and

ABI5 control mainly seed related ABA responses (Finkelstein *et al.* 1998, Finkelstein and Lynch 2000, Parcy *et al.* 1994).

Dormancy is initiated early during seed maturation and continues until the seed is fully mature (Raz *et al.* 2001). In Arabidopsis, the seed maturation and induction of dormancy is mainly controlled by four transcription factors namely *FUSCA3* (*FUS3*), *ABSCISIC ACID INSENSITIVE 3* (*ABI3*), *LEAFY COTYLEDON 1* (*LEC 1*) and *LEC 2* (Baumlein *et al.* 1994, Giraudat *et al.* 1992, Lotan *et al.* 1998, Stone *et al.* 2001). *ABI3*, *FUS3* and *LEC2* encode transcription factors containing the conserved B3-binding domain that are plant specific (Giraudat *et al.* 1992, Luerssen *et al.* 1998, Stone *et al.* 2001), whereas *LEC1* encodes a HAP3 subunit of the CCAAT-binding transcription factor CBF (Lotan *et al.* 1998). All four transcription factor mutants *abi3*, *lec1*, *lec2* and *fus3*, affect seed maturation severely and share some common phenotypes, such as decreased dormancy at maturation (Raz *et al.* 2001) and reduced expression of seed storage proteins (Gutierrez *et al.* 2007). A study, using Arabidopsis cultivars that differed in dormancy, showed no correlation between *LEC1*, *FUS3*, *ABI3* and *Em* expression and dormancy (Baumbusch *et al.* 2004). Although all four genes affect embryo maturation, they also play a unique role in regulating each other's functionality and expression pattern (Holdsworth *et al.* 2008b). *FUS3* controls formation of epidermal cell identity and embryo derived dormancy (Tiedemann *et al.* 2008). Loss of *LEC1* causes germination of excised embryos similar to *lec2* and *fus3* mutants (Raz *et al.* 2001). *LEC2* controls the transcription program during seed maturation and one of the genes that is affected by it is *DELAY OF*

GERMINATION 1 (*DOG1*), which is the first seed dormancy gene responsible for variation that occurs in natural environment identified by QTL analysis (Bentsink *et al.* 2006, Braybrook *et al.* 2006). Both *LEC1* and *LEC2* regulate the expression of *FUS3* and *ABI3* (Kagaya *et al.* 2005, Kroj *et al.* 2003). In addition, *ABI3* and *FUS3* positively auto-regulate themselves and each other creating a feedback loop (To *et al.* 2006). Interestingly, none of these four transcription factors (*LEC1*, *FUS3*, *ABI3* and *LEC2)* contains motifs to interact with an ABA response element (ABRE), but do contain a B3 domain that interacts with the RY motif present in the promoters of genes that produce RNA during the late maturation phase of the seed (Braybrook *et al.* 2006, Ezcurra *et al.* 1999, Monke *et al.* 2004, Reidt *et al.* 2000). The Arabidopsis *ABSCISIC ACID INSENSITIVE 5* (*ABI5*) is a basic leucine zipper (bZIP) domain-containing protein that is a transcription factor interacting with ABRE and activating ABA-mediated transcription in seeds (Carles *et al.* 2002, Finkelstein and Lynch 2000). ABI3, which activates RY elements, physically interacts with ABI5 and this physical interaction seems to be necessary for ABA-dependent gene expression (Nakamura *et al.* 2001).

Although much information on dormancy regulation is available for dicots like Arabidopsis, the molecular control of dormancy in cereals is not very clear. One of the key genes in regulating seed maturation, dormancy and desiccation in maize is *Viviparous1* (*VP1*), an ortholog to *ABI3* in *Arabidopsis* (Giraudat *et al.* 1992, McCarty *et al.* 1989, McCarty *et al.* 1991). It is also responsible for transcriptional control of the *LATE EMBRYOGENESIS ABUNDANT* (*LEA*) class of proteins (Nambara *et al.* 1995, Nambara *et al.* 2000). *VP1* is also involved in

root growth-related crosstalk between ABA and auxin (Suzuki *et al.* 2001). Quantitative Trait Loci (QTL) analysis showed *VP1* to be responsible for seed dormancy and PHS (Flintham *et al.* 2002, Lohwasser *et al.* 2005). *VP1* is responsible for controlling embryo maturation and dormancy as well as inhibition of germination (Hoecker *et al.* 1995, McCarty *et al.* 1991). Like ABI3, VP1 interacts with ABI5 to regulate embryonic gene expression and seed sensitivity to ABA (Lopez-Molina *et al.* 2002). *VP1/ABI3* has been cloned from various dicot and monocot species (Hattori *et al.* 1994, Jones *et al.* 1997, Rohde *et al.* 2002) and contains a N-terminal acidic domain (A1) and three basic domains designated B1, B2 and B3 (Giraudat *et al.* 1992). The A1 domain is responsible for ABA-mediated transcriptional activation, B2 for ABRE-mediated transcriptional activation and B3 for RY/G-box interaction (Ezcurra *et al.* 1999, Hoecker *et al.* 1995). *VP1/ABI3* is also alternatively spliced in various plant species and its mis-splicing causes PHS in wheat (Gagete *et al.* 2009, McKibbin *et al.* 2002, Wilkinson *et al.* 2005). *ABI5* undergoes alternative splicing forming two variants which interact with each other and each having distinct binding affinity to *VP1/ABI3* (Zou *et al.* 2007). In barley, ABA-dependent up-regulation of *ABI5* is positively regulated by a feed-forward mechanism that involves *ABI5* itself and *VP1* (Casaretto and Ho 2005). These reports suggest a very prominent role of transcriptional regulation in fine tuning ABA responses.

2.3.2 Inhibition of GA signalling by DELLA proteins

Genes encoding protein components of GA signalling have been proposed to effect a regulatory role on seed germination (Peng and Harberd 2002). Inside the nucleus, the DELLA proteins, a family of putative transcriptional regulators, mediate the GA signal (Dill *et al.* 2001, Itoh *et al.* 2002, Richards *et al.* 2000, Wen and Chang 2002). DELLA proteins inhibit GA responses and thus are considered negative regulators of GA signal transduction (Wen and Chang 2002). Arabidopsis has five DELLA genes (*GA-INSENSITIVE* [*GAI*], *REPRESSOR OF GA1-3* [*RGA*], *RGA-LIKE1* [*RGL1*], *RGL2*, and *RGL3*), while rice *SLENDER1* (*SLR1*) and other species such as barley *SLENDER1* (*SLN1*), maize, and wheat have only one DELLA protein (Chandler *et al.* 2002, Dill *et al.* 2001, Itoh *et al.* 2002, Peng *et al.* 1999). Downstream of the DELLA proteins, GA regulates α-amylase synthesis in aleurone layer tissue via a Myb-like transcription factor (GAmyb) that binds to a specific region of the promoter of genes that encode α-amylase (Gubler *et al.* 1995). The GA-signal is perceived by a soluble GA receptor with homology to human hormone-sensitive lipase, *GA-INSENSITIVE DWARF1* (*GID1*) (Ueguchi-Tanaka *et al.* 2005). The binding of bioactive GAs to GID1 promotes an interaction between GID1 and the DELLA-domain of DELLA protein (Ueguchi-Tanaka *et al.* 2007, Willige *et al.* 2007), which in turn enhances the affinity between DELLA-GID1-GA complex and a specific SCF E3 ubiquitin–ligase complex, SCFSLY1/GID2, involving the F-box proteins AtSLY1 and OsGID2 in Arabidopsis and rice, respectively (Griffiths *et al.* 2006, McGinnis *et al.* 2003, Sasaki *et al.* 2003, Willige *et al.* 2007). The SCFSLY1/ GID2 promotes the

ubiquitinylation and subsequent destruction of DELLAs by the 26S proteasome (Fu *et al.* 2002, McGinnis *et al.* 2003, Sasaki *et al.* 2003). The *DELLA* genes are transcriptionally controlled by the light-labile transcription factor PIL5 which promotes the transcription of *GAI* and *RGA* genes by directly binding to their promoters through the G-Box (Oh *et al.* 2007).

GA-dependent degradation of DELLA protein is inhibited by ABA in barley and by both ABA and salt (NaCl) in Arabidopsis (Achard *et al.* 2006, Gubler *et al.* 2002). In Arabidopsis, the two independent salt-activated phytohormonal signalling pathways (ABA and ethylene) regulate plant development through integration at the level of DELLA function (Achard *et al.* 2006). DELLA also affects flowering in an ABA-dependent manner (Achard *et al.* 2006); however, its function in regulation of dormancy and germination is not clear. Germination in tomato, soybean and Arabidopsis is not dependent on down-regulation of DELLA genes (Bassel *et al.* 2004). Arabidopsis *sly1* mutant seeds can germinate despite a high content of RGL2 protein, the DELLA protein that specifically represses seed germination (Ariizumi and Steber 2007). Far-red light is known to inhibit germination through DELLA dependent induction of ABI3 activity and ABA biosynthesis while DELLA-mediated cotyledon expansion breaks coat-imposed dormancy (Penfield *et al.* 2006, Piskurewicz *et al.* 2009).

2.4 ABA-mediated epigenetic regulation of genes

Despite the lack of complete information about ABA signalling, it is well established that ABA responses are regulated by transcriptional regulation, except for the quick responses in stomatal closure (Wasilewska *et al.* 2008).

Recent discoveries reveal that besides transcriptional regulation, ABA mediates epigenetic regulation to control plant responses (Chinnusamy *et al.* 2008). ABA-mediated epigenetic regulation of gene expression in seeds is now being studied extensively. Polycomb group-based gene imprinting and DNA methylation/demethylation control seed development in plants (Eckardt 2006). Seed specific physiological processes like dormancy and germination are being studied in the context of epigenetic regulation. A cDNA-AFLP-based study showed epigenetic regulation of transcripts during barley seed dormancy and germination (Leymarie *et al.* 2007). Gene expression during seed development and germination inhibition are also regulated by ABA through networks of transcription factors belonging to the B3 domain (ABI3, VP1, LEC2, FUS3), APETELA2 (ABI4), bZIP (ABI5) and HAP3 subunit of CCAAT binding factor (LEC1) (Finkelstein *et al.* 2002). ABA regulates the B3 domain transcription factors through *PICKLE* (*PKL*) which encodes putative CHD3 type SWI/SNF-class chromatin-remodeling factor (Ogas *et al.* 1999). ABA-mediated stress responses occur through Histone Deacetylase (HDACs)-dependent chromatin modifications and ATP-dependent chromatin remodelling complexes that include SWI3-like proteins (Rios *et al.* 2007, Wu *et al.* 2003). Stress-related memory also appears to be inherited through epigenetic mechanisms (Boyko *et al.* 2007). ABA also regulates non-coding small RNAs (siRNA and miRNA) that can induce epigenetic changes through DNA methylation (Bond and Finnegan 2007, Yang *et al.* 2008).

2.5 Common mechanisms that regulate flowering and bud dormancy

The first clue regarding the commonality between factors controlling flowering and bud dormancy arose from environmental signals that regulated them (Chouard 1960). The signalling events responsible for regulation of flowering and bud dormancy converge on *FLOWERING LOCUS T* (*FT*) (Bohlenius *et al.* 2006). Day length is an important determinant in regulation of flowering acting through its photoreceptor *PHYTOCHROME A* (*PHYA*). PHYA affects the floral induction pathway through its effect on *CONSTANS* (*CO*), a gene involved in flowering pathway, which in turn affects *FT* (Yanovsky and Kay 2002). *FT* is negatively regulated by *FLC* which regulates temperature-dependent seed germination in Arabidopsis (Chiang *et al.* 2009, Helliwell *et al.* 2006). *FCA* and *FVE* regulate *FT* under high and low temperatures in a *FLC*-dependent manner (Blazquez *et al.* 2003, Sheldon *et al.* 2000). Although limited, the information regarding the intricate network of signalling events that regulate the two most important events, namely the transition from vegetative to reproductive state, and from non-germinated to germinated state suggests some common factors (Horvath 2009).

2.6 Introduction to FCA

The RNA-binding protein FCA regulates transition to flowering in Arabidopsis (Koornneef *et al.* 1991, Macknight *et al.* 1997). The expression of *FCA* has so far been studied in leaves and flower buds under short and long day conditions (Macknight *et al.* 1997). Post-transcriptional regulation of *FCA*, a

process conserved in Arabidopsis (dicot) and rice (monocot), occurs through alternative splicing and alternative polyadenylation resulting in four different transcripts (α, β, γ and δ) of which only γ forms a functional protein (Lee *et al.* 2005, Macknight *et al.* 1997). *FCA* α is the least abundant isoform (<1%) followed by δ (10%), γ (35%) and β (55%). The FCA protein consists of two RNA recognition motifs (RRMs) and a protein interactor WW domain (Macknight *et al.* 1997). FCA is auto-regulated by its own transcript levels through its interaction with FY, a 3'-end processing factor (Quesada *et al.* 2003). In Arabidopsis the regulation of *FCA* transcript levels by interaction of FCA and FY is an important determinant in regulating FLC and controlling floral transition (Simpson 2004). FCA also localized to its target FLC chromatin suggesting the regulation of FLC by FCA is through chromatin modification (Liu *et al.* 2007a). FCA acts with small interfering RNA to mediate chromatin silencing through methylation of various single and low copy gene loci in the Arabidopsis genome (Baurle *et al.* 2007). However, no apparent difference in DNA methylation at the *FLC* locus were observed between *fca* mutants and wild-type plants (Veley and Michaels 2008).

A recent study regarding interaction of FCA and FY show that the interaction between the two is only transient and that the FY may be the key factor in regulating the RNA processing machinery in Arabidopsis (Manzano *et al.* 2009). FCA–FY interaction is required for general chromatin silencing roles where hairpin transgenes induce DNA methylation of an endogenous gene (Baurle *et al.* 2007). The two RNA processing factors AtCPSF100 and AtCPSF160 (Zhao *et al.* 1999), but not FCA, are stably associated with FY *in vivo* and form a range of

different sized complexes (Manzano *et al.* 2009). A hypomorphic *fy* allele, producing a shorter protein which was able to provide some FY functions but unable to interact with FCA, reduced the abundance of some of the larger MW complexes. Suppressor mutants, which specifically disrupt the FY motif through which FCA interacts, also lacked these larger complexes. The authors suggested that FCA, perhaps after recognition of a specific RNA feature, transiently interacts with FY, an integral component of the canonical RNA 3' processing machinery, changing the interactions of the different RNA processing components (Manzano *et al.* 2009).

The effect of *fca* mutation on cell fate and cell autonomy of Arabidopsis seeds showed that *fca* mutants had no defect in cell number or cell fate in the dry seed except for the fact that it led to the production of more rosette leaves (Furner *et al.* 1996). The mutation of the *fca* gene in regions L_2 and L_3, regions in the shoot apical meristem that are surrounded by layer L_1, did not result in late bolting suggesting that the existence of diffusible signals from layer L_1 expressing normal *FCA* lead to a normal bolting phenotype (Furner *et al.* 1996).

In Arabidopsis, FCA interacts with AtSWI3B a homologue of core subunit of a yeast chromatin remodelling complex (Sarnowski *et al.* 2002). Modification in chromatin structure plays an important role in gene regulation (Li *et al.* 2007). The relationship between various kinds of histone modifications and between histone modification and DNA methylation remains unclear (Veley and Michaels 2008).

The study of conservation and divergence of functions of FCA in Arabidopsis and rice suggest that the splicing mechanism and the effects on flowering are conserved between the two but the mechanism of auto-regulation of splicing and flowering control may be different (Lee *et al.* 2005). The interaction between rice homologues of FCA and FY is also conserved (Lu *et al.* 2006). Rice *FCA* over-expressed in Arabidopsis did not result in reduction of *FCA*-γ transcripts in the transgenic plants (Lee *et al.* 2005). Rice *FCA* could partially rescue the late flowering phenotype of Arabidopsis *fca-1* mutant, a known target of Arabidopsis *FCA* that controls flowering in Arabidopsis (Lee *et al.* 2005). Constitutive expression of *rFCA-RRM2* containing the two RRM and a WW domain delayed flowering in transgenic rice and increased grain weight up to fifty percent (Attia *et al.* 2005).

The interaction of FCA and FY has mainly been implicated in floral transition but the possibility of the existence of additional targets other than floral transition has not been ruled out (Marquardt *et al.* 2006). The expression of the poplar homologue of *FCA* that is up-regulated when apical dormancy is triggered hints towards additional role(s) of *FCA* in induction of dormancy (Ruttink *et al.* 2007).

3. MATERIALS AND METHODS

3.1 Plant material, treatment and growth conditions

3.1.1 Barley

Barley, *Hordeum vulgare* L. cv. McLeod was used for germination assays as well as cloning and characterization of *FCA*. Seeds of cultivar McLeod were obtained from Brian G. Rossnagel (University of Saskatchewan) from either the 2004 or 2007 harvest. The 2004 seeds of cultivar McLeod had higher level of dormancy and ABA responsiveness compared to 2007 harvest (Fig 4.9 A, 4.10 A and appendix 6). Transient expression assays were performed on McLeod embryos and promoter GUS assays and α-amylase assays were performed on Himalaya aleurone layer protoplasts (1993 harvest, University of Washington, Pullman, USA). All the seeds were stored at -20°C.

For mature embryo studies, seeds were surface sterilized with 50% (v/v) bleach for 15 min. and then washed 3-4 times with autoclaved, deionised water. Seeds were imbibed in water or ABA for 45 min. and embryos were isolated with a scalpel. The seed coat was cut on the dorsal side of the grain just below the embryo keeping the scalpel at an angle so that the embryo came out clean without any endosperm attached to the scutellar surface. Care was taken to minimize aleurone layer tissue contamination. Embryos were then placed in water, ABA or NaCl for incubation for different time periods. Incubated embryos stored at -78°C until used. A racemic mixture of ABA (±ABA) was used for all experiments.

For seedling studies, McLeod seeds were surface sterilized and allowed to grow in half-strength Murashige and Skoog basal salt mix (MS medium) (Sigma) up to the single leaf stage in a growth chamber under 16/8 hrs. day/night period at 25°C. The light intensity was maintained at 120 µmol/m^2sec. Seedlings were then transferred to half-strength MS medium containing the desired concentrations of NaCl or ABA. Samples were then collected and stored at -78°C until used.

3.1.2 Arabidopsis

Arabidopsis *fca-1* mutant line and wild-type plants, both in Landsberg background, were obtained from the Arabidopsis Biological Resource Center, Columbus, Ohio, USA. The *fca-1* plants transformed with *FCA* gene under a constitutive 35S promoter (modified pBI121) were obtained from Divya Rajagopal, (post doctoral fellow, Robert Hill's lab, University of Manitoba).

The seeds from Arabidopsis *fca-1* mutant transformed with barley *FCA* were screened on half-strength MS media plates containing 1% (w/v) sucrose, 1% (w/v) agar and kanamycin (50mg/L). The seeds were sterilized by washing in 70% (v/v) ethanol for 1 min. followed by a solution containing 50% (v/v) bleach and 0.5% (w/v) SDS for 5 min., ending with 3-4 rinses in sterile water. The seeds were kept on kanamycin plates and stratified for 3 days at 4°C in dark. After stratification, they were placed in a growth cabinet for 10 days before transferring them into soil under the same growth conditions (16 hrs. light, 22°C temperature, 120 µmol/m^2sec light intensity). The Arabidopsis lines showing 3:1 ratio for

resistance : susceptible for kanamycin were chosen for generating homozygous plants. The homozygous lines thus obtained were used for further studies.

3.2 RNA extraction, quantification and cDNA synthesis

Two methods of RNA extraction were tested. A guanidine-HCl based method gave good quality and yield of RNA but DNA contamination was an issue and the samples had to be treated with DNase before cDNA synthesis or Northern blotting. A TRI® reagent (Sigma)-based method was quick with very little or no DNA contamination and hence was used for all the experiments. Approximately 200 mg tissue were used to isolate total RNA according to the manufacturer's instructions. For real-time PCR studies, total RNA was treated with RNase-Free DNase (Qiagen) according to manufacturer's instructions and further purified using RNeasy mini kit (Qiagen) according to manufacturer's instructions. Messenger RNA (mRNA) extraction was performed with PolyATtract® mRNA Isolation System IV (Promega) according to the manufacturer's instructions. Approximately 100-150 µg total RNA were required to yield 1-1.5 µg of mRNA.

The quantity and purity of extracted nucleic acids in solution (DNA and RNA) were spectrophotometrically determined by measuring the absorbance at 260 nm and 280 nm. A value of OD_{260} of 1 corresponds to 50 µg/ml for double stranded DNA, while OD_{260} of 1 corresponds to 40 µg/ml for RNA. For DNA and RNA, $OD_{260/280}$ ratios between 1.8 and 2.0 were deemed acceptable (Maniatis and Sambrook 1982).

First strand cDNA synthesis was performed with 5 µg of total RNA, oligo dT (18-22 bp) primers and Superscript II reverse transcriptase (Invitrogen) or M-MLV reverse transcriptase (Promega), following manufacturer's protocols. After the first strand synthesis, the reaction was terminated by heat inactivation at 70°C for 10 min.

3.3 Gene Cloning

3.3.1 Primer designing and Polymerase Chain Reaction (PCR)

For PCR amplification, specific or degenerate primers were designed. The melting temperature (Tm) of the primers was calculated according to Faust rules, Tm (°C) = 4 (G+C) +2 (A+T) where G, C, A and T represent the number of corresponding nucleotides in the primer. The annealing temperature was set at Tm ± 5°C. The primers were designed to avoid self-complementation leading to formation of secondary structures. The forward and reverse primers for each reaction were designed to have approximately the same Tm.

Based on barley *FCA* EST sequences (TC160622) and a wheat *FCA* sequence, various primers were designed to amplify the barley *FCA*. Forward (5'- GTC AAA ACC CTA GCC ACC TTC - 3') and reverse (5'- CCA CCT GCA GTC AAC TTT TCC - 3') primer combination gave the complete coding region of barley *FCA*. A 3' Rapid Amplification of Complimentary Ends (RACE) was performed with forward primer (5' – TCA GCT GTA CAG TCC AAT CCC ATG – 3') and the adapter reverse from the Genome Walker Universal Kit (Clontech Laboratories, Inc.) to amplify approximately 1.1 kb fragment containing the complete 3' UTR.

For the amplification of DNA fragments, a solution containing 15 pmol/µl each of both forward and reverse primers, 5 ng/µl plasmid DNA or PCR product or 2-5 µl of first strand cDNA as a template was mixed and the volume was made to 50 µl with the Platinum® PCR SuperMix (Invitrogen) in a sterile 0.2 ml thin-wall PCR tube (Fisher Scientific). PCR reactions were performed in a PCR Sprint Thermal Cycler (Thermo Electron Corporation) or a PTC-100 Thermal Cycler (MJ Research Inc.). The standard reaction conditions used were: initial denaturation at 95°C for 5 min., followed by 34 cycles of 95°C for 1 min., 64°C for 45 sec, 72°C for 3 min. and a final extension of 20 min. at 72°C. An aliquot from the amplified mix was electrophoresed on a 1% (w/v) agarose gel with a standard molecular weight marker (1 Kb Plus DNA Ladder, Invitrogen) to check for amplification. The right size fragment, usually a single amplicon, was gel extracted using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. The gel-extracted and purified DNA fragments were cloned into a pGEM-T Easy vector (Promega) according to the manufacturer's instructions.

3.3.2 Preparation of *E. coli* competent cells, transformation and plasmid isolation

A single colony of *E.coli* DH5α cells was inoculated into 5 ml of LB broth (Bertani 1951) and incubated overnight with constant shaking at 37°C. One ml of the overnight culture was grown in 50 ml of LB medium with vigorous shaking until the OD_{600} reached 0.5. The cells were cooled on ice for 10 min. and pelleted by centrifugation at 4000 rpm for 5 min. at 4°C. The pellet was suspended in 40

ml of ice-cold 100 mM $CaCl_2$, incubated on ice for 20 min., and further centrifuged as above. The pellet was finally resuspended in 3 ml ice-cold 100 mM $CaCl_2$, 50% (v/v) sterile glycerol, mixed and stored at -70°C in aliquots of 0.2 ml of competent cells.

For transformation, 5-10 µl of the plasmid DNA (10-50 ng /µl) or the ligated plasmid DNA construct were added to competent cells, mixed with a pipette and incubated on ice for 30 min. The cells were subjected to heat shock at 42°C for 90 sec., and then placed on ice for 2 min. LB medium (0.8 ml) was added to the treated cells and further incubated at 37°C with shaking at 200 rpm. Aliquots (100-200 µl) of the transformed cells were spread on selection plates and incubated at 37°C overnight. Individual colonies were selected and were inoculated in LB liquid media with appropriate antibiotic and cultured overnight. An aliquot of 0.5 ml of the culture solution from each colony was mixed with 50% glycerol and stored at -78°C. Rest of the culture was used for plasmid isolation using GenElute™Plasmid Miniprep Kit (Sigma). The isolated plasmids were confirmed for the presence of insert by digestion with appropriate restriction enzyme. DNA digestion was carried out in a reaction volume of 50 µl containing the appropriate reaction buffer and 5 U of restriction enzyme per 1 µg of DNA to be digested. The plasmids containing the insert were confirmed by sequencing.

3.4 Construction of vectors for plant transformation and transient expression studies

Different constructs were prepared for full length *FCA* and truncated *FCA* (without RRM) for transient expression and localization studies. For transient expression studies, pBluescript (SK) vector was modified by inserting the maize *ubiquitin* promoter and *nos* terminator in the multiple cloning sites. The modified pBluescript (SK) vector plasmid was restricted with BamHI to clone *FCA*/truncated *FCA* in sense and anti-sense orientations. Single digestion product of the modified vector was dephosphorylated to avoid self-ligation of cohesive termini of plasmid DNA during DNA recombination. DNA dephosphorylation was carried out with calf intestine alkaline phosphatase (Roche) according to manufacturer's protocol. The reaction was made in a total volume of 50 µl comprising 5 µl dephosphorylation buffer (10 times buffer provided with the kit), 1 µl (1.0 unit) calf intestine alkaline phosphatase and appropriate µg of plasmid DNA. The mixture was made up to 50 µl with sterile distilled water. The reaction mixture was incubated at 37°C for 1 hr. and followed by heat inactivation at 65°C for 15 min. and further purified by QIAquick Gel Extraction Kit (Qiagen).

The pGEM-T Easy plasmid containing the full length *FCA* was restriction digested with BamHI and the *FCA* fragment was gel purified. The purified *FCA* was then ligated into modified pBluescript (SK) vector, transformed into DH5α and multiple colonies were selected based on antibiotic resistance. Plasmids were isolated from the selected clones and were checked for sense and anti-sense orientation of the insert by performing PCR using *ubiquitin* promoter

forward (designed at the 3'-end) and gene reverse primers. The clones found positive for presence of insert in either orientation were confirmed by sequencing. Truncated *FCA* (without RRM) was amplified by primers (forward 5'- GCA CTT GCA GCC ATG AAT TCT C -3'; reverse 5'-GCT GAC ACA TCA TTC ATC CAC CTG C -3') and cloned in the same vector digested with BamH1 under the control of a *ubiquitin* promoter and *nos* terminator.

For localization studies, full length *FCA* was cloned into pDONR 221 (Invitrogen) and then transferred into the pEarlygate destination vector CD3 683 containing YFP-HA tag at the C-terminal according to manufacturer's protocol. The truncated *FCA* (without RRM) construct was prepared by cloning the cDNA into a modified version of pRTL2/GFP (Hwang *et al.* 2004), donated by Doug Muench, University of Calgary, that had the *RFP* coding sequence inserted in place of the *GFP* coding sequence. A construct, donated by R.S. Quatrano, University of Washington, containing *VP1* gene fused to GFP under the control of the ubiquitin promoter was used as a control for nuclear localization of protein in aleurone layer protoplasts (Marella and Quatrano 2007).

3.5 Sequence and phylogenetic analysis

The confirmed plasmids harbouring the desired DNA fragments were analyzed using the BLAST search (NCBI). Multiple sequence alignments were carried out with nucleotide sequences retrieved from the NCBI GenBank database, TIGR database and Computational Biology and Functional Genomics laboratory databases (http://compbio.dfci.harvard.edu/tgi/). A tree was

constructed by multiple alignments of nucleic acid sequences in DNAMAN bioinformatic analysis software (Lynnon Corporation).

3.6 Extraction of RNA and Northern blot analysis

3.6.1 Extraction of total RNA

Total RNA was extracted from the frozen samples prepared from different abiotic stress-treated and from unstressed samples using TRI® reagent (Sigma) according to the manufacturer's instructions. Approximately 100-150 µg total RNA were used to extract messenger RNA (mRNA) by PolyATtract® mRNA Isolation System IV (Promega) according to its technical manual. The RNA samples were prepared by adding 1 volume of 5x RNA loading buffer per 4 volumes of RNA sample (for example: 10 µl of 5x RNA loading buffer and 40 µl of RNA). This was incubated for 3-5 min. at 65°C, chilled on ice and loaded onto a gel. RNA loading buffer (5x) was prepared by mixing saturated bromophenol blue -16 µl, 0.5 M EDTA, pH 8.0 - 80 µl, 37% formaldehyde (12.3 M) -720 µl, 100% (v/v) glycerol - 2ml, formamide - 3084 µl, 10x formaldehyde gel buffer - 4ml and RNase-free water to 10 ml.

3.6.2 Extraction of messenger RNA and its immobilization on membranes

Messenger RNA (1 µg) was separated on a 1.2% (w/v) formaldehyde agarose gel (1.2 g of agarose, 10 ml 10x MOPS buffer, RNase-free water to 100 ml were added and agarose was melted in microwave; then cooled to 65°C, added 1.8 ml of 37% (v/v) (12.3 M) formaldehyde and 1µl of ethidium bromide (10

mg/ml), mixed thoroughly and poured onto gel support) by electrophoresis at 90 V for 2-3 hrs. in 1X formaldehyde gel buffer (100 ml 10x 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, 20 ml 37% (v/v) formaldehyde (12.3 M) made up to 1 L with RNase-free water; MOPS Buffer (10x): 200 mM MOPS (free acid), 50 mM sodium acetate, 10 mM ethylenediaminetetraacetic acid (EDTA) to pH 7.0 with sodium hydroxide. The gel was washed in diethylpyrocarbonate (DEPC) water for 30 min. The DNA was transferred onto Hybond-N+ nylon membrane (Amersham Biosciences) overnight by capillary method using 20X SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) as a transfer buffer. The mRNA transferred on the membrane was UV cross-linked according to manufacturer's instructions.

3.6.3 Generation and radio-labelling of DNA probes

Barley *FCA* probes were generated through PCR of *FCA* cDNA with forward (5' – TTTTTGGATCCCGGCTCAGTTGTTTCATGG G – 3') and reverse (5'–TTTTGGATCCACGTCTGCTGTGAAGATTGATAA C–3') primer combinations from a region between the RRM and WW domain. The protocol follows the method of the Prime-a-gene Labeling System (Promega). In a 1.5 ml microcentrifuge tube, 25 ng of the denatured template DNA, 10 µl labeling buffer (5x), 2 µl of a mixture of unlabeled dNTPs (dGTP, dATP and dTTP), 2 µl of nuclease-free BSA, 5 µl of 250 µCi/µl [α-^{32}P] dCTP (Invitrogen) and 5 units of DNA polymerase I (Klenow) fragment were added with the volume of the reaction mixture adjusted to 50 µl. This mixture was then incubated at room temperature

for 1-2 hrs. The reaction was terminated by heating at 95-100°C for 5 min. and subsequently chilled on ice and used directly in a hybridization solution.

3.6.4 Hybridization of DNA probe with immobilized mRNA

The membrane containing the cross linked mRNA was pre-hybridized at 65°C for 1-2 hrs. in phosphate buffer (0.5 M phosphate buffer, pH 7.2, 7% (w/v) SDS, 10 mM EDTA and 1% (w/v) BSA) and hybridized for 24 hrs. with 50 µl of radiolabelled DNA probe (see chapter **3.6.3**).

After hybridization, the membranes were washed twice with 2X SSC, 0.1% (w/v) SDS at 65°C for 10 min., followed by 1X SSC, 0.1% (w/v) SDS and 0.1X SSC and 0.1% (w/v) SDS for 5 min. each respectively. The membranes were exposed at -78°C to X-Ray flim (Kodak) or Clear Blue X-Ray film (Thermo Scientific) (Church and Gilbert 1984). All glassware, plasticware, buffer solutions used for RNA work were treated with 0.1% (v/v) DEPC and autoclaved.

3.7 Protein isolation and Western blot analysis

3.7.1 Total protein isolation from plant tissue

Embryo and aleurone layer samples were prepared from mature barley seeds. After incubation with water or 10 µM ABA for 24 hrs., the samples were air-dried and collected tissue was immediately frozen in liquid nitrogen and stored at -78°C until used. The tissue was ground to a fine powder with a pestle in a pre-chilled mortar. Microsomal fractions were obtained by homogenizing ground tissue in homogenization buffer (100 mM MES buffer, pH 5.5 (5 ml g^{-1}), containing

250 mM sucrose, 3 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM freshly prepared dithiothreitol). The homogenate was filtered through four layers of cheesecloth and centrifuged twice for 10 min. (15,000x *g*) at 4°C in a Sorvall RC6 Plus centrifuge. The filtrate was centrifuged at 111,000x *g* for 65 min. (4°C) in a Sorvall Discovery 120 FE ultracentrifuge, and the pellet containing crude microsomal fractions were collected. Cytosolic proteins were obtained from the 111,000x*g* supernatant. The protein concentration was measured by Bradford's method (Bradford 1976).

3.7.2 Purification of recombinant ABAP1 protein

A 250 ml culture of the bacterial clone containing the gene expressing His-tagged ABAP1 was grown for 16 hrs. in an incubator shaker at 37°C. When the OD_{600} reached between 0.6-0.8, 250 µl of 0.5 M isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the culture was kept on the incubator shaker at 37°C for 4 hrs. The culture was then centrifuged for 10 min. at 6000 rpm and the collected pellet was treated with 3 ml lysis buffer containing 50 mM NaH_2PO_4 (pH 7.4), 300 mM NaCl, 10 mM imidazole, 0.5 mM PMSF and 5% (v/v) glycerol. The pellet was vortexed to dissolve and then it was sonicated 10 times for 30 sec. at 30 sec. intervals. Since ABAP1 is known to be insoluble or precipitates in the solution, 0.4% (w/v) sodium dodecyl sulfate (SDS) was added to keep the protein in solution. The sonicated solution was then centrifuged at 7800 rpm for 15 min. at 4°C and the supernatant was collected. The Ni-NTA agarose beads (Qiagen) were normalised with lysis buffer (8 times lysis buffer to

bead volume) and the supernatant protein was mixed with nickel beads. The proteins were allowed to bind to the nickel column by mixing them on a rotor at 4°C for 1 hr. The beads were then washed with 5 volumes of wash buffer (50 mM NaH_2PO_4 (pH 7.4), 300 mM NaCl, 50-100 mM imidazole); the protein eluted in batches of 0.5 ml volumes with a total of 3 ml elution buffer (50 mM NaH_2PO_4 (pH 7.4), 300 mM NaCl, 300 mM imidazole).

3.7.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-polyacrylamide gel (12% or 15% (w/v)) electrophoresis was performed according to the protocol of (Laemmli 1970). Samples were boiled at 95°C for 5 min., cooled in ice and loaded on the gel. The gels were run on a Mini PROTEAN Tetra Gel System (Bio-Rad) at 50 V until the proteins were stacked properly and thereafter gels were run at a constant 100 volts. The gels were stained with 0.25% (w/v) Coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid.

3.7.4 Western blotting

The proteins were transferred to polyvinylidine fluoride (PVDF) Millipore Immobilon-P membrane using a Mini PROTEAN Tetra Gel System (Bio-Rad) and allowed to dry at 4°C for 16 hrs. The blots were blocked for 60 min. at room temperature in blocking buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05 Tween 20, and 5% (w/v) milk powder) and washed with washing buffer (Tris-buffered saline, 0.05% (v/v) Tween 20). The blots were then incubated with AB2

antibodies (1:1000 dilution of 1.12 mg/ml) for 60 min. at room temperature (Razem *et al.* 2004). Blots were washed twice for 10 min. followed by a 15 min. wash in washing buffer and subsequently incubated with secondary antibodies (1:1000 dilution, anti-mouse conjugated with alkaline phosphatase) (Sigma) for 60 min. The membrane was washed with washing buffer (3 times, 10 mins each) and immersed in staining buffer containing nitroblue tetrazolium (5% w/v) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (5% w/v) in alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) for 10 min. before the reaction was stopped by washing in double distilled water, and the blots were left to dry overnight at room temperature.

3.8 Isolation of aleurone layer protoplasts and protoplast transformation

3.8.1 Isolation of aleurone layer protoplasts

The aleurone layer protoplasts were isolated according to the protocol of (Jacobsen *et al.* 1985). Barley quarter seeds were treated with 3.5% NaOCl for 15 min. and washed with de-ionised distilled water. Twenty-five quarter seeds were placed into sterile 25 ml Erlenmeyer flasks containing 1 ml of 50 mM arginine solution containing 50 mM arginine hydrochloride, 20 mM $CaCl_2 \cdot 2H_2O$, 150 µg/ml timentin and 250 IU/ml nystatin. The flasks were plugged loosely with cotton wool and left at room temperature for three days. The quarter of the seeds were then removed from the arginine solution and the starchy endosperm was removed by scraping with spatulas under sterile conditions. The aleurone layers obtained were transferred into sterile 25 ml Erlenmeyer flasks containing 1.5 ml protoplast

isolation medium (2% (w/v) glucose, 10 mM arginine hydrochloride, 4.5% (w/v) Onozuka cellulase RS, 1% (w/v) PVP K25, 10mM MES, 0.35 M mannitol, 20 mM $CaCl_2 \cdot 2H_2O$, 150 µg/ml timentin, 500 IU/ml nystatin) prepared with Gamborg's B-5 medium with minimal organics (Sigma). Isolation medium was removed and 1 ml of incubation medium containing 0.67M mannitol, 2% (w/v) glucose, 10 mM arginine hydrochloride, 150 µg/ml timentin and 500 IU/ml nystatin prepared in Gamborgs's solution was added. The flask was gently swirled to free the protoplasts from digested aleurone layers and the aleurone tissue debris was removed by tweezers. The protoplast solution thus obtained was cleaned by Percoll gradient centrifugation. The gradient consisted of 7 ml each of 15% (v/v) and 80% (v/v) Percoll (80% Percoll with arginine solution; 15% by dilution of 80% with incubation media) at room temperature. The protoplast suspension was loaded onto the gradient and centrifuged at 600 rpm in a SS-34 rotor in Sorvall RC 6 Plus centrifuge for 15 min. with acceleration and deceleration set at 5. Recovered protoplasts from the interface of the 15% and 80% Percoll gradient were transferred to a 50-ml sterile disposable tube and the volume adjusted to 50 ml with incubation medium. The solution was mixed gently and centrifuged as before. The supernatant was discarded and the protoplasts were used for transformation. Quality and yield of the protoplasts was checked under a compound microscope with 20X magnification.

3.8.2 Protoplast transformation

For protoplast transformation (Gubler and Jacobsen 1992), incubation medium was removed and the protoplasts were suspended in MaMgMES solution (0.5 M mannitol, 15mM $MgCl_2$, 0.1% (w/v) MES balanced pH to 5.6 with NaOH) in sterile 50 ml tubes. The volume was adjusted depending upon the number of transfection treatments (2 ml times the number of transfections). Within 20 min. of adding MaMgMES, calf thymus DNA (200 µg/ml protoplast) and 4 µg each of the plasmids containing genes of interest were added to the protoplast solutions and mixed gently for 1 min. Polyethylene-glycol-containing (PEG) solution (40% (w/v) PEG 4000, 0.5 M mannitol, 0.1 M Ca $(NO_3)_2.4H_2O$ pH adjusted to 9.0) was added (300µl/transformation, filter sterilized) to the protoplast solution, mixed gently and incubated at room temperature for 30 min. After incubation, 300 µl of 0.2 M $CaCl_2$ were added to the solution, mixed gently and incubated for 45 min. Protoplasts were then centrifuged at 500 rpm for 5 min. and supernatants were removed. Incubation medium (3 ml, filter sterilized) was used to re-suspend the protoplasts and 1.5 ml of each suspension were transferred to 25 ml flasks for 48 hrs. under different treatments (water and ABA). After 48 hrs., protoplasts were harvested by transferring them into 1.5 ml tubes and spun at 600 rpm for 5 min. Incubation medium was stored for assay of α-amylase and the protoplasts were lysed with 200 mM Tris buffer (pH 7.0). Protoplast extracts were analyzed for luciferase and GUS activity.

3.9 β-Glucuronidase (GUS) and Luciferase (LUC) assay

Transformed protoplasts were harvested by centrifugation at 600 rpm for 5 min. and lysed using Tris buffer (0.25 M, pH 5.7). A GUS assay was performed by the protocol mentioned in (Gomez-Cadenas *et al.* 1999). Protoplast extracts (100 µl) were used for the GUS assay using 4-methylumbelliferyl-β-D-glucuronide (4-MUG) containing GUS assay buffer (50mM sodium phosphate buffer (pH=7.0), 10mM EDTA (pH=8.0), 0.1% w/v sodium lauryl sarcosine, 0.1% (v/v) TritonX-100, 1mM 4-MUG, 10mM β-mercaptoethanol (β-ME) (added fresh) and 20% (v/v) methanol). Fluorescence was measured with excitation at 365nM (slit width=15) and emission at 455nm (slit width=20) in a Perkin-Elmer Luminesence Spectrophotometer. A luciferase assay was performed with a LUC assay kit (Promega) as per the manufacturer's instruction using a Berthold Technologies Junior LB 9509 Luminometer. A standard curve for 4-MU activity was prepared by measuring the fluorescence of various concentrations of 4-MU (0-500 ηM) prepared in 1 ml of stop solution (Na_2CO_3) (Appendix 1).

3.10 Transient expression using biolistic particle delivery system

A gold particle suspension was prepared by mixing 0.5 g gold particle (spherical, < 10 micron) (Sigma) in 1ml of absolute ethanol. It was vortexed 3 times for 30 sec. each and allowed to sit at room temperature for 10 min. The suspension was then centrifuged at 10,000 rpm for 1 min. and the supernatant was discarded. 500 µl of absolute ethanol was added to the gold pellet and vortexed. This gold suspension was stored at -20°C.

For mature embryo bombardment studies, 2 μg of plasmid were used for every shot unless stated otherwise. The plasmids were precipitated onto the gold particles (100 μg/shot) by adding 2.5 M $CaCl_2.2H_2O$ (10 times the volume of plasmid solution) and 0.1 M spermidine (4 times the volume of plasmid solution) while constantly vortexing the tubes. The plasmid gold mixture was incubated at 20°C for 2 hrs. to enhance precipitation and then centrifuged at 10,000 rpm for 10 sec. The resulting pellet was washed with 250 μl of absolute ethanol and centrifuged again at 10,000 rpm for 10 sec. The supernatant was removed and the pellet containing the plasmid coated gold particles was brought into suspension with absolute ethanol (20 μl/shot) depending upon the number of shots.

The gold particles coated with plasmids were bombarded onto 25-30 embryos (scutellum side up) with a Biolistic® PDS-1000/He particle delivery system (Biorad). A 20 μl aliquot of gold plasmid mix was added onto the micro-carrier and allowed to dry. The biolistic system was set up according to manufacturer's instructions. A rupture disc of 2000 psi, vacuum of 15 inches of Hg and a target distance of 6 cms were used to bombard embryos. Bombarded embryos were then incubated in treatments of water or ABA.

3.11 Real-time quantitative PCR and analysis of relative gene expression

Real-time PCR was performed on cDNA samples for different target genes with actin as reference. Gene specific primers (*HvFCA*: Forward: 5'- AGGCTC AAACAATTCCATCTA-3';Reverse:5'-TCAACTTTTCCAAGAACGCTCT-3';

H*vVP1*: Forward: 5'-TCTCGATCCCCATTGAGGAC -3'; Reverse: 5'-CTTTAC ATCGGAGTAGAGCAC -3'; *HvABI5*: Forward: 5'- GCAAGCAGGCTTATACAA TGG -3'; Reverse: 5'- TCACCAGGGCCCGGTCAG -3') were used to amplify a portion of the gene using ABI 7500 Fast Real-Time PCR System (Applied Biosystems). For PCR, 2-4 µl of the reverse transcription reaction were used as cDNA template, 0.5 µl of forward and reverse primer mix (100mM), 12.5 µl of 2X Quantifast SYBR green mix (Qiagen) and the volume adjusted to 25 µl with water. The amplification was performed with cycling conditions of 95° for 5 min. and 40 cycles between 95° for 45 sec. (denaturation), 62°C for 45 sec. (annealing and extension). Fluorescence was measured at the annealing and extension step. The reverse transcription PCR on Arabidopsis plants were performed with primers: (*actin*: Forward: 5'- AGG GAG AAG ATG ACT CAG ATC -3'; Reverse: 5'-GTG TGA GAC ACA CCA TCA CC-3'; *FLC*: Forward: 5'-CTG TTC TCT GTG ACG CAT CC -3'; Reverse: 5'-GTG TGA ACC ATA GTT CAG AGC -3'). The data obtained from real-time PCR was analysed from the mean of triplicates by the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen 2001).

3.12 Sub-cellular localization studies

For localization studies in onion epidermal cells, constructs were precipitated on gold particles and bombarded onto the chopped onion tissue (see section 3.10 for Biolistic-mediated transient expression). The bombarded tissue was incubated for 24 hrs. before microscopy. The microscopy was performed on a VanGuard microscope (model no.1486 FL) fitted with 100W mercury lamp and

YFP excitation (500/25nm) and emission filters (530 nm). The image was captured through a Nikon Coolpix 8800 camera.

Protoplasts were transformed (see section 3.8.2) with various constructs and incubated in incubation medium for 18 hrs. before confocal microscopy. An Olympus IX70 confocal microscope equipped with Uplan Apo X40 dry objective, filters for RFP and GFP, differential interference contrast (DIC) optics, 100W mercury arc light source, Argon and Krypton laser source was used for imaging RFP and GFP signal. Excitation and emission wavelengths were set at 563/582 nm for RFP and 385/508 nm for GFP. The images were acquired in XY plane (2D) in the FLOWVIEW 2.1 software.

3.13 Chemicals, kits and services used

3.13.1 Chemicals used

Chemicals used were obtained from Fisher Scientific and Sigma (USA) unless otherwise stated. Cellulase “Onozuka” R-10 and RS were obtained from Yakult Honsha Co.Ltd., Japan.

3.13.2 Source of kits used during study

Kits used during study were obtained from: QIAGEN Plasmid Midi Kit & Qiagen Plasmid Mini Kit (Qiagen), QIAquick Gel Extraction Kit (Qiagen), Prime-a-Gene Labeling System (Promega), RNeasy Mini Kit (Qiagen), PolyATtract® mRNA Isolation System IV (Promega), QuantiFast SYBR Green PCR kit (Qiagen), TRI Reagent RNA Isolation Reagent (Sigma), Luciferase Assay System

(Promega), GenomeWalker Universal Kit (Clontech Laboratories, Inc.), QIAexpress Type IV kit and Ni-NTA magnetic beads (Qiagen).

3.13.3 Modifying enzymes and molecular markers

Modifying enzymes and molecular markers used during the study were obtained from: Alkaline Phosphatase (Roche), DNase (RNase-Free, Qiagen), DNA Ligase (Invitrogen), Gateway BP Clonase II enzyme mix & Gateway LR Clonase II enzyme mix (Invitrogen), M-MLV Reverse Transcriptase (Promega), RNasin (Promega), Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen), GoTaq Flexi DNA Polymerase (Promega), Phusion High-Fidelity DNA Polymerase (New England Biolabs), Restriction Enzymes (Promega), 1kb Plus DNA marker (Invitrogen), 100 BP DNA ladder (New England Biolabs), RNA markers (0.28 - 6.5 kb)(Promega), Rainbow Protein MW markers (Amersham).

3.13.4 Custom primer synthesis

Primers used in this study were custom synthesized from Invitrogen, USA.

3.13.5 Custom sequencing

All PCR fragments and recombinant clones were sequenced by MacrogenUSA.

3.14. Statistical analysis

Statistical analysis using paired t-test was performed on sigma stat software. The transient expression studies on barley aleurone layer protoplasts were done in triplicates. The transient expression studies on barley embryos were done in triplicates and the experiment was repeated twice. The results presented are from triplicates of the representative experiment. The results were considered significant at $p<0.05$ (5% significance level).

4.0 RESULTS

4.1 Cloning of barley *FCA* and its relationship to *ABAP1*

4.1.1 Cloning of barley *FCA*

The *ABAP1* gene was cloned from a barley aleurone layer and was shown to encode an ABA binding protein sharing significant homology to FCA at the C-terminal region (Razem *et al.* 2004). There have, however, been concerns regarding the independent existence of *ABAP1* (Finkelstein 2006). To investigate if *ABAP1* is actually a partially-cloned fragment of barley *FCA*, 5'- Rapid Amplification of Complementary Ends (RACE) was performed. Despite repeated attempts, the *ABAP1* transcript could not be extended completely in the 5' region via RACE. Meanwhile, barley *FCA* was identified as a collection of ESTs (TC160622) that had significant homology to rice (AY274928) and wheat (AY230398) *FCA*. Based on the sequence information from barley *FCA* ESTs, primers were designed to amplify the full length open reading frame of *FCA* from barley embryos yielding an amplification product of 2.3 kb (Fig. 4.1 A). A 3'-RACE was performed using a forward primer from within the gene and an adapter reverse primers (supplied with the genome walker kit, Clontech) to amplify the complete 3'UTR up to the poly A tail (Fig. 4.1 B). Translation of the open reading frame and analysis of the putative amino acid sequence indicated that the protein consists of 743 amino acids harbouring regions rich in glycine residues, two RNA Recognition Motifs (RRMs), a WW protein interaction domain and the poly-glutamine domain (Fig. 4.1 C). The complete cDNA sequence (Fig. 4.1 D) was submitted to NCBI (FJ188402).

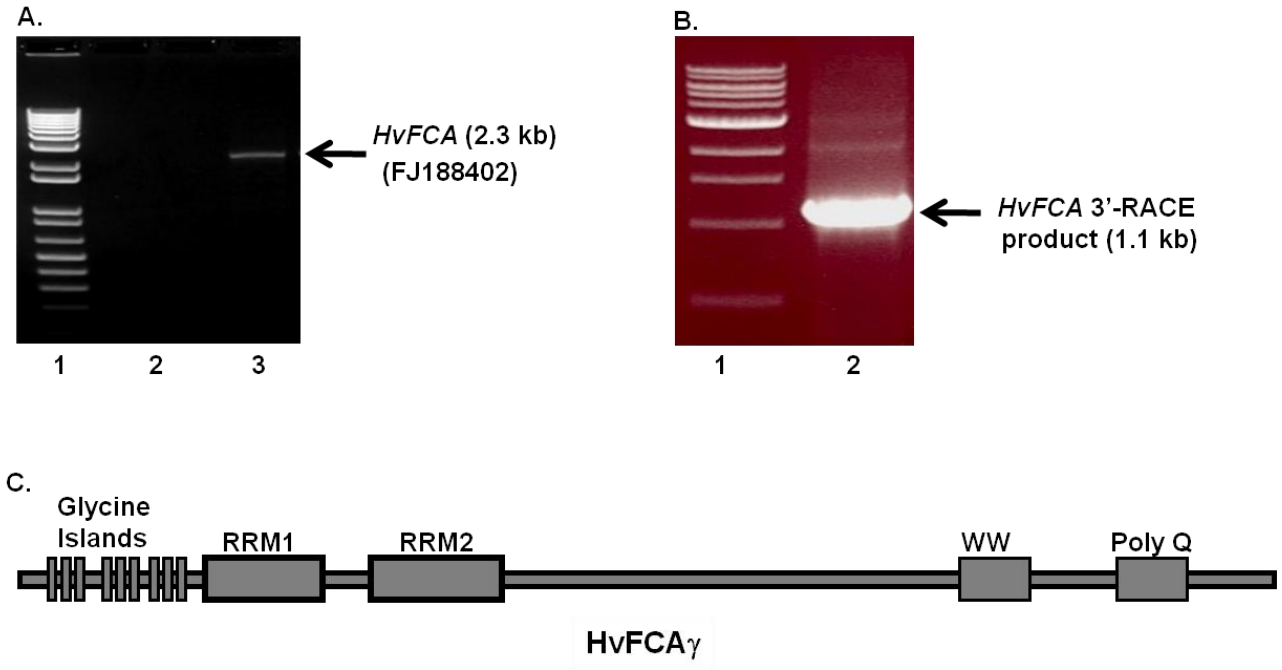


Figure 4.1. Analysis of the products of PCR amplification to identify the sequence of barley *FCA*. **(A)** Agarose gel electrophoresis of RT-PCR-amplified band derived from barley mRNA using primers to amplify *FCA* ORF. Lanes 1: 1kb plus DNA ladder, 2: empty, 3: barley *FCA* fragment. **(B)** Agarose gel electrophoresis of RT-PCR band derived from 3'-RACE performed to complete the ORF of barley *FCA*. Lane 1: Lambda DNA *EcoRI* + *HindIII* ladder, 2: 3'-RACE product. **(C)** Diagrammatic representation of barley *FCA*(γ) to indicate regions containing various domains like RNA Recognition Motifs (RRM), protein-protein interaction domain (WW), glycine rich repeats and a polyglutamine domain (polyQ).

TTAAGTCAATCCGCCCGGCGCCAGGCGGCCGCGGGAATCGATTGTCAAAACCCTAGCCACCTTCTGCCTCCA
CATGCACCGCGGCAGCGACCGCTCCGCCGACCCTTCAGGCCCCGCGGGCGCCGCCCGCAGCGGCGGGGACGG
CAGATTCGCGCGCGGACCCTCACGCTGGTCCGGCGGCGGCGGGGGTAGCCCCCCGCCCCACCGCTCCTCCCG
CGGCGGGAGCAGCGACGGCGGCGGAGGCGGAGGCGGAGGCGGAGGCGGGAGGCTCCATCCGTACCGTGCGCC
GTCTGAATACGTTGTTGGCGGTGGTGGAACTGGAGGGTACAGAGGCGGCGGCGGCGACTTTGATGAGACGGC
CGGTGGAGCGAAGAGCCGTTACGGAGGCGGTGGCGGCGGAGGCCGTGGAGACTACTCAGATCATGATAACAA
AAGTGGTTATGTTAAACTTTTCGTTGGATCAGTTCCAAGAACAGCAAATGAAGATGATGTTCGACCTTTATT
TGAGGATCATGGAGATGTTCTTGAAGTTGCTTTGATCAGGGATAGGAAAACTGGTGAACAACAAGGCTGTTG
TTTTGTTAAATATGCTACTTCCGAAGAGGCCGAGAGAGCCATCAGAGCTCTGCATAACCAGTGCACTATACC
CGGGGCGATGGGCCCTGTTCAGGTTAGATATGCTGATGGTGAAAAGGAGCGTCATGGGTCTATTGAGCACAA
ATTGTTTGTTGCATCACTGAATAAGCAGGCAACTGCAAAGGAGATAGAAGAGATTTTTGCTCCTTTTGGTCA
TGTGGAAGATGTTTACATCATGAAAGATGGCATGAGGCAGAGCCGAGGCTGTGGCTTTGTCAAATTCTCATC
AAAAGAACCTGCACTTGCAGCCATGAATTCTCTTAGTGGGACTTACATAATGAGGGGGTGCGAGCAACCATT
AATAGTTCGATTTGCTAATCCTAAGAGGCCTAGACCTGGAGAATCAAGGGGTGGCCCTGCCTTCGGAGGTCC
TGGTGTCAGTTCTCGATCTGATGCAGCACTAGTTATCAGGCCGACTGCCAATCTTGATGAGCAAATAGGTCG
ACACATGCCTCCTGACACTTGGCGTCCTTCAAGCCCAAGCTCAATGGCACCTCATCAGTTCAATAACTTCGG
GTCTGACAATTCTATGGGCCTGATGGGTGGCCCTGTTACATCAGCAGCAGATAATGTTGCTTTTCGGCCTCA
GTTGTTTCATGGGAATGGTTCTTTGTCAAGTCAGACAGCTGTGCCGGCATCGTCTCATATGGGCATAAATCC
TTCCTTGTCACAAGGGCATCATCTCGGTGGGCCACAGATCCCACCCTTGCAAAAGCCAACTGGCCTGCAGCA
GAATTTCCCTGTACAATTGCAGAATGCTCAGCAAGGGCAGCTTCATGCCTCACAATCCTTGGGGCCTGGTTC
TTTTGGCCAGAATATACCAACTATGCAATTACCTGGCCAGCTCCCTGTGTCACAACCATTGACGCAGCAAAA
TGCTTCTGCATGCGCTCTACAGGCGCCTTCAGCTGTACAGTCCAATCCCATGCAATCTGTTCCTGGACAACA
ACAACTTCCATCCAATTTAACACCACAAATGCTACAGCAGCCAGTCCAGCAGATGCTGTCACAAGCTCCACA
GTTGCTACTCCAACAGCAGCAGGCAGCTATGCAGTCCAGTTATCAATCTTCACAGCAGACGATTTTTCAGCT
TCAGCAACAGCTGCAACTAATGCAGCAGCAGCAGCACCAGCAGCAGCCTAACTTAAATCAGCAGCCACATAC
GCAGGTTCCTAAGCAACAGGGACAGCCAGTGCAATCTAATGCCCCTGGTGCTCCGGCTGCCATGATGACGAC
AAACATAAATGCAATTCCACAGCAGGTCAATTCGCCTGCAGTTTCTTTAACTTGCAATTGGACAGAACATAC
CTCACCTGAAGGTTTTAAATATTACTACAATAGCATAACTCGAGAGAGTAAGTGGGAAAAGCCTGAAGAATA
TGTACTGTATGAGCAGCAGCAGCAGCAGCAGCAGCACCAGAAACTTATTTTACTTCAACAGCACCAACAAAA
GCTTGTTGCGCAGCAACTTCAGTCACCTCCTCAGGCTCAAACAATTCCATCTATGCAATCTATGCAACACCA
TCCCCAGTCGCAGCAAGGACATAACCAAATGCAGATGAAACAGCAGGATTTAAACTATAATCAGTTACAGCC
AACGGGCACGATTGATCCCAGTAGGATTCAGCAGGGAATTCAAGCTGCTCAAGAGCGTTCTTGGAAAAGTTG
ACTGCAGGTGGATGAATGATGTGTCAGCGAAGACTCCAGTCTCAGGAATGAGCTCCAGCAAGACCTGCCGCT
TCTGCCTGTGACGGTGTTTTTTGCCTTCGCGCGGATGGCCATGTTGGCCCTTGCGGTCATTGTAACTCTGAA
TTTAGCTTAGATTAGTGCCTAGATTGTAGATCCGATGTGTGTAAAATGTTTGCAGTCTAGGCCTTGTATCGC
TGTAACATTGCCTATTAGAATGGCAGCTGTGTGTCGCTGTAACATTCAGTGTTTTTATCTACCTTTTTTATG
GCCAGAGTTGCCGTCTCATATGCATTAGCATTGCTATTAAAAAAAAAAAAAAAAA

Figure 4.1. (D). Complete open reading frame coding sequence of barley *FCA.* The portion of the sequence with black background represents start codon (ATG) and stop codon (TGA). The grey background portion of the sequence represents the 3' untranslated region (UTR).

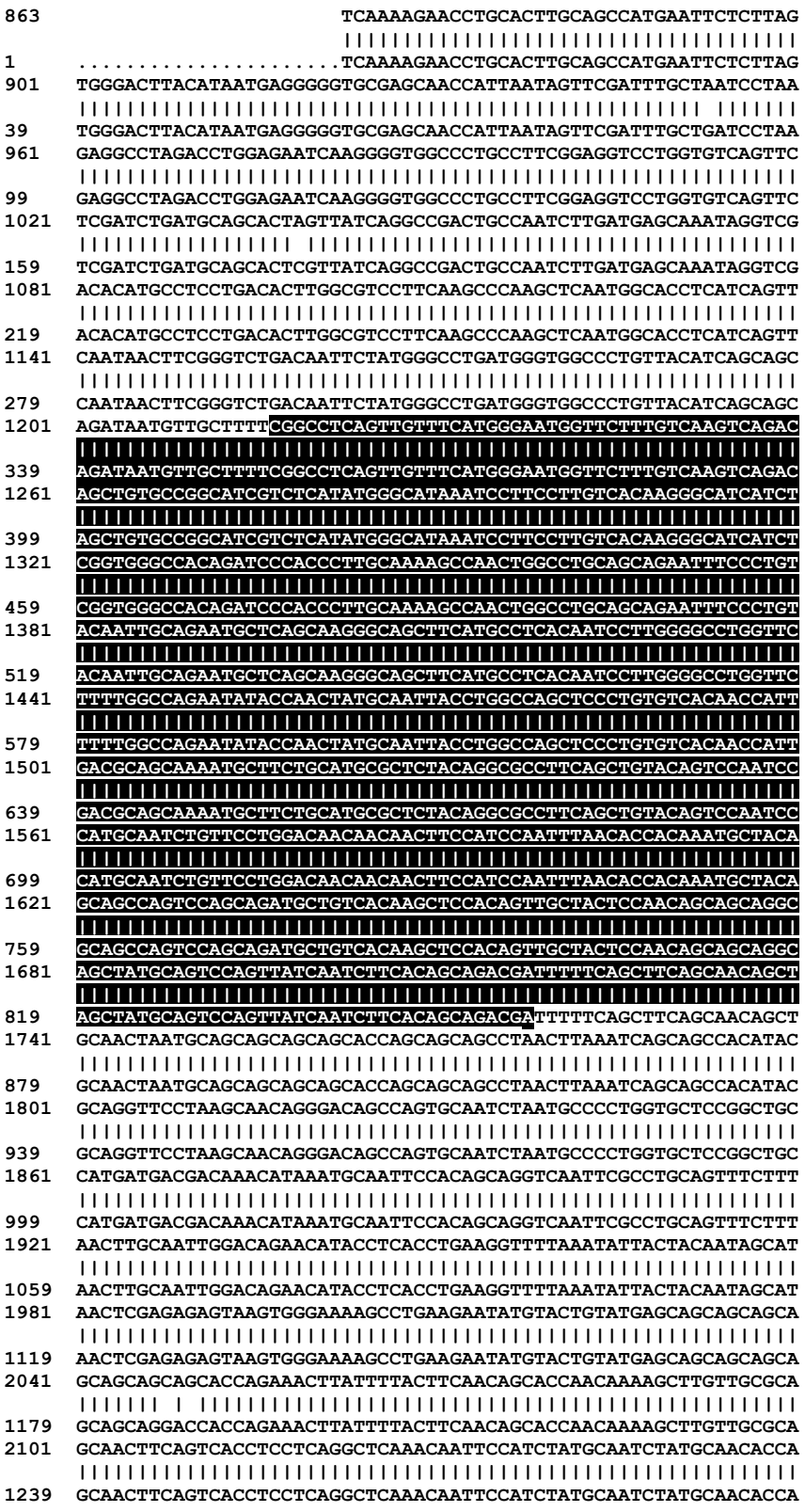
4.1.2 Relationship between *ABAP1* and *FCA*

Barley *ABAP1* and *FCA* sequence similarity was analysed using DNAMAN software. The two sequences were identical throughout the *ABAP1* sequence region (Fig. 4.2 A). Northern blots were carried out on mRNA isolated from barley embryos, incubated in the absence or presence of 10µm ABA for 24 hrs., or from dry embryos to check for the existence of *FCA* and *ABAP1* as independent transcripts. A 500 bp probe was designed from the region between the RRM and WW domain that is common to *FCA* and *ABAP1* (Fig. 4.2 A & B). Since the transcript length of *FCA* and *ABAP1* are almost identical to 28S RNA and 18S RNA, the Northern blot was carried out on mRNA, taking care to remove total RNA contamination. The probe detected a single fragment migrating at the size of *FCA* (2.7 kb) but no transcripts were detected for a size corresponding to *ABAP1* (1.7kb) (Fig. 4.3).

It is suggested that there are two copies of the *ABAP1* gene in the barley genome (Razem *et al.* 2004). The possibility that *ABAP1* arises from a different genomic region compared to *FCA* was examined. The genomic region upstream of *ABAP1* was amplified from cultivar McLeod embryo genomic DNA, based on the genome walking sequence information obtained from Dr. Fathey Sarhan's lab (University of Montreal, unpublished result). A sequence similarity search based on nucleotide blast (NCBI) analysis showed that the genomic DNA fragment obtained from the 5'-region of *ABAP1* (1.7 kb) had 100% similarity to expressed regions of barley *FCA* (FJ188402.2) (Appendix 2). This sequence also showed significant similarity to wheat and rice *FCA*.

A.

Alignment of *HvFCA* (upper line) and *ABAP1* (lower line).



```
2161  TCCCCAGTCGCAGCAAGGACATAACCAAATGCAGATGAAACAGCAGGATTTAAACTATAA
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1299  TCCCCAGTCGCAGCAAGGACATAACCAAATGCAGATGAAACAGCAGGATTTAAACTATAA
2221  TCAGTTACAGCCAACGGGCACGATTGATCCCAGTAGGATTCAGCAGGGAATTCAAGCTGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1359  TCAGTTACAGCCAACGGGCACGATTGATCCCAGTAGGATTCAGCAGGGAATTCAAGCTGC
2281  TCAAGAGCGTTCTTGGAAAAGTTGACTGCAGGTGGATGAATGATGTGTCAGCGAAGACTC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1419  TCAAGAGCGTTCTTGGAAAAGTTGACTGCAGGTGGATGAATGATGTGTCAGCGAAGACTC
2341  CAGTCTCAGGAATGAGCTCCAGCAAGACCTGCCGCTTCTGCCTGTGACGGTGTTTTTTGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1479  CAGTCTCAGGAATGAGCTCCAGCAAGACCTGCCGCTTCTGCCTGTGACGGTGTTTTTTGC
2401  CTTCGCGCGGATGGCCATGTTGGCCCTTGCGGTCATTGTAACTCTGAATTTAGCTTAGAT
      |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
1539  CTTCGCGCGGATGGCCATGTTGGCTCTTGCGGTCATTGTAACTCTGAATTTAGCTTAGAT
2461  TAGTGCCTAGATTGTAGATCCGATGTGTGTAAAATGTTTGCAGTCTAGGCCTTGTATCGC
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1599  TAGTGCCTAGATTGTAGATCCGATGTGTGTAAAATGTTTGCAGTCTAGGCCTTGTATCGC
2521  TGTAACATTGCCTATTAGAATGGCAGCTGTGTGTCGCTGTAACATTCAGTGTTTTTATCT
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1659  TGTAACATTGCCTATTAGAATGGCAGCTGTGTGTCGCTGTAACATTCAGTGTTTTTATCT
2581  ACCTTTTTTATGGCCAGAGTTGCCGTCTCA
      ||||||||||||||||||||||||||||||
1719  ACCTTTTTTATGGCCAGAGTTGCCGTCTCA
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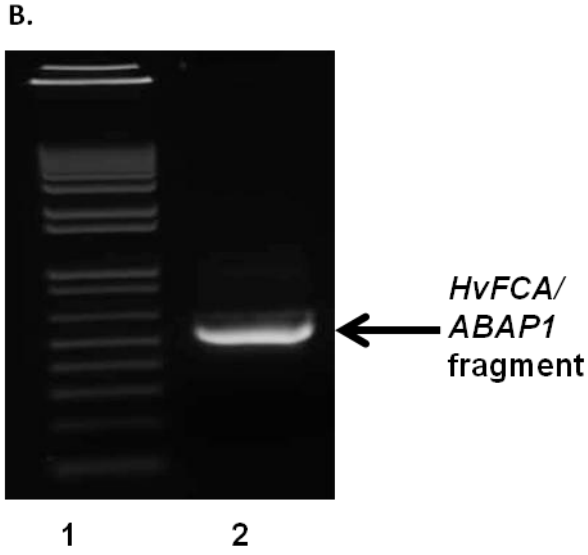


Figure 4.2. (A) Nucleotide sequence similarity (shown by vertical lines) between barley *FCA* and *ABAP1*. The shaded area represents the sequence common to both *FCA* and *ABAP1* that was used for generation of probes for Northern blot analysis. **(B)** Gel picture showing PCR amplification of the barley *FCA*/*ABAP1* fragment used as DNA probe. Lanes 1: 1kb plus ladder, 2: barley *FCA*/*ABAP1* fragment.

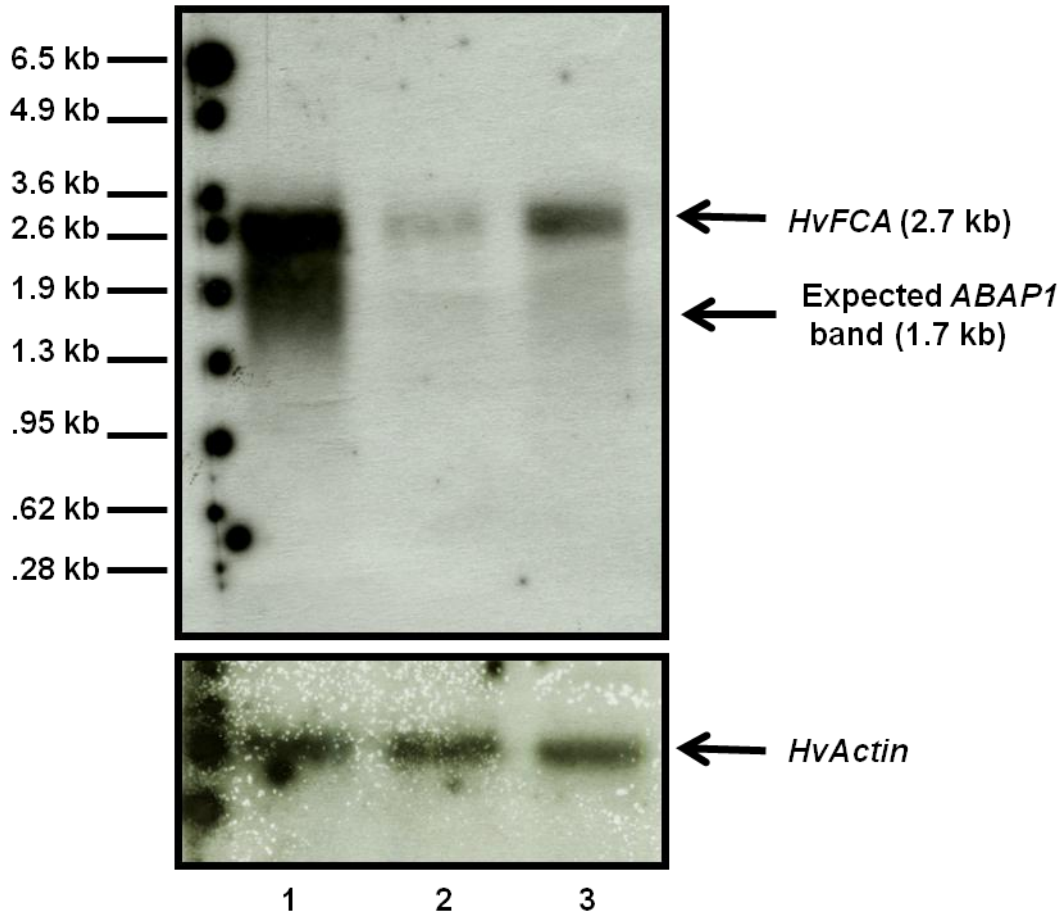


Figure 4.3. Northern blot of barley (2007 harvest) embryo mRNA probed with *FCA/ABAP1*. Lanes 1: dry mature embryo, 2: water treated (24 hrs.) embryos, 3: embryos treated with ABA (10µm) (24 hrs.). Approximately 1 µg of mRNA free of total RNA contamination was loaded into each well.

In the initial experiments describing ABAP1, Western blots using an anti-idiotypic antibody raised against (±) ABA monoclonal antibody designed to identify ABA-binding proteins showed the presence of ABAP1 as an independent protein in ABA-treated barley aleurone layer tissues (Razem *et al.* 2004). A recombinant ABAP1 expressed in *E. coli* was used as a positive control. Attempts were made to repeat this work. Proteins extracted from cytosolic as well as microsomal fractions of water- and ABA- (10µM) treated barley aleurone tissues were extracted for Western blotting using the same anti-idiotypic antibody. The binding specificity of the monoclonal antibody was assayed using the recombinant ABAP1 protein with and without a HIS tag. No protein of the appropriate molecular size reacted with the antibody in the cytosolic or microsomal fraction of the water- or ABA-treated barley aleurone layer protein (Fig. 4.4), at least at the level of detection of the antibody, i.e., approaching 10 ng or considerably less than 0.04% percent of the total aleurone protein loaded. The recombinant protein without HIS tag migrated faster on SDS polyacrylamide gel compared to the one with HIS tag attached and in both cases the anti-idiotypic antibody recognized the recombinant ABAP1 protein. Similar experiments were also conducted using an antibody raised against recombinant ABAP1. The anti-ABAP1 antibody detected the recombinant protein at the right size (52 kDa) (Fig. 4.5 A) but also detected other non-specific low molecular mass proteins. Western blotting on different barley tissue also detected multiple fragments of varying sizes including a protein fragment at 52 kDa in embryo and leaf protein extracts of barley (Fig. 4.5 B). The identity of this 52 kDa protein detected by anti-ABAP1 antibody is unknown.

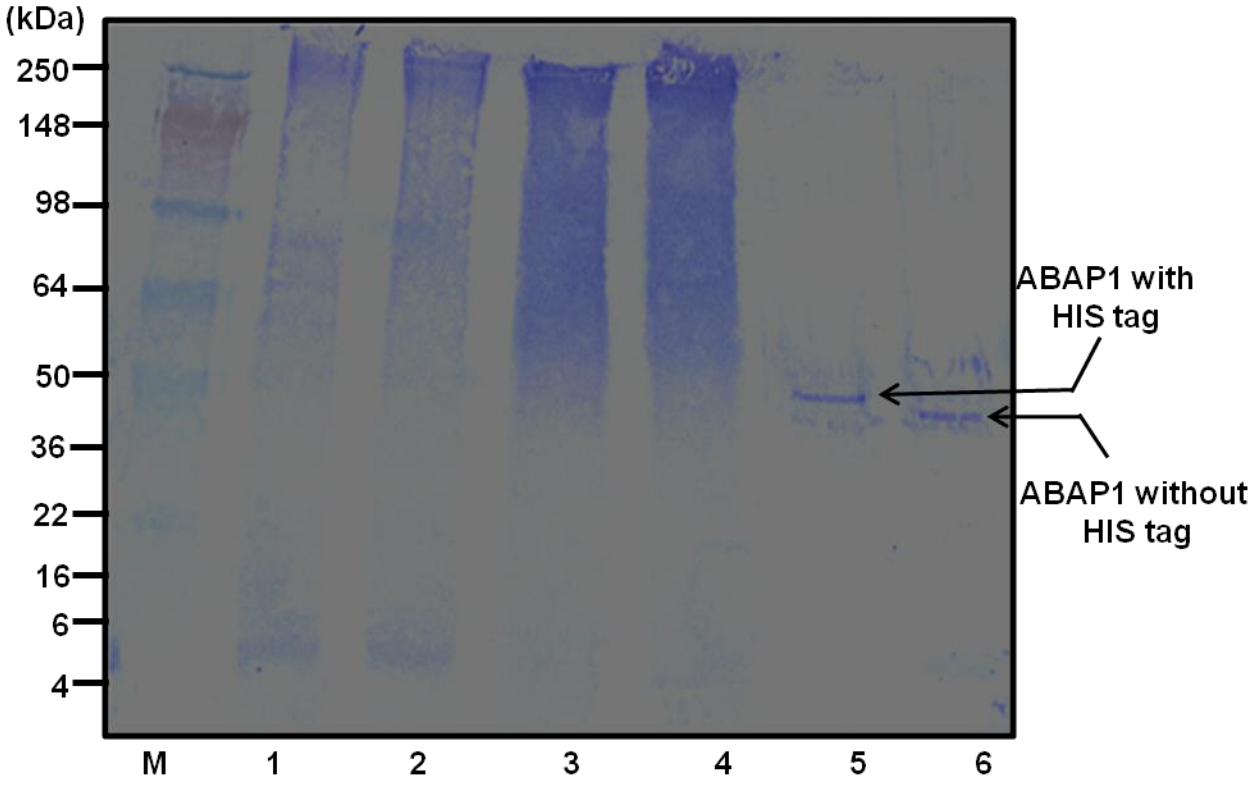


Figure 4.4. Western blot performed on cytosolic and microsomal fractions of water- and ABA-treated (24 hrs) barley aleurone layer protein. Protein loads were 25µg for aleurone layer proteins and 12.5 ng for recombinant protein. Lanes: M: SeeBlue Plus2 pre-stained protein standard, 1: cytosolic protein (H_20), 2: cytosolic protein (10 µM ABA), 3: microsomal protein (H_20), 4: microsomal protein (10 µM ABA), 5: non-restricted recombinant ABAP1 protein and 6: recombinant ABAP1 protein without HIS tag. AB2 antibodies were used as primary antibody (1:1000 dilution) and an anti-mouse conjugated with alkaline phosphatise was used as secondary anti-body (1:1000 dilution).

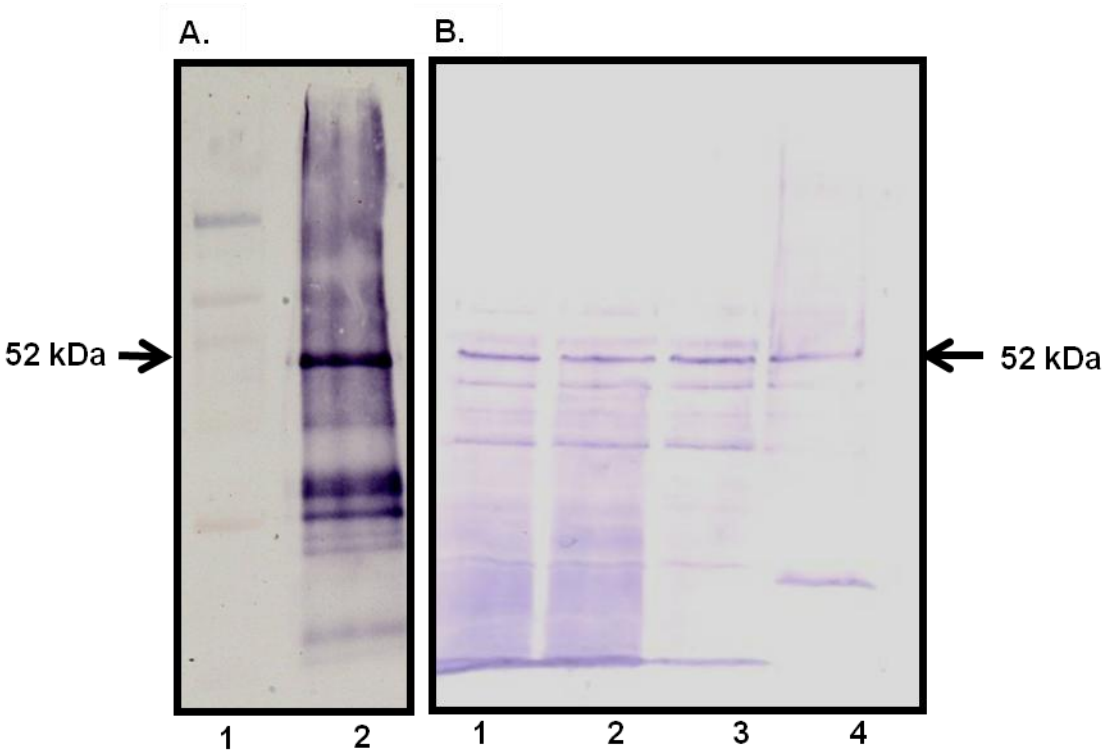


Figure 4.5. Western blotting of recombinant ABAP1 protein (with HIS tag) and of total protein extracts from barley. Anti-ABAP1 antibody serum was used as a primary antibody (1:4000) an anti-mouse conjugated with alkaline phosphatase was used as secondary antibody (1:1000 dilution). **(A)** Western blot of recombinant barley ABAP1 (with His tag). Lanes: 1: Rainbow MW markers high range (Amersham), 2: recombinant ABAP1. **(B)** Western blot on total protein (10 µg) fraction of 24 hour water- and ABA-treated barley embryos and barley leaves. Lanes: 1: water treated embryo protein, 2: embryo protein (10 µM ABA), 3: embryo protein (10 µM GA) and 4: water treated 5 day old first green leaf tissue proteins.

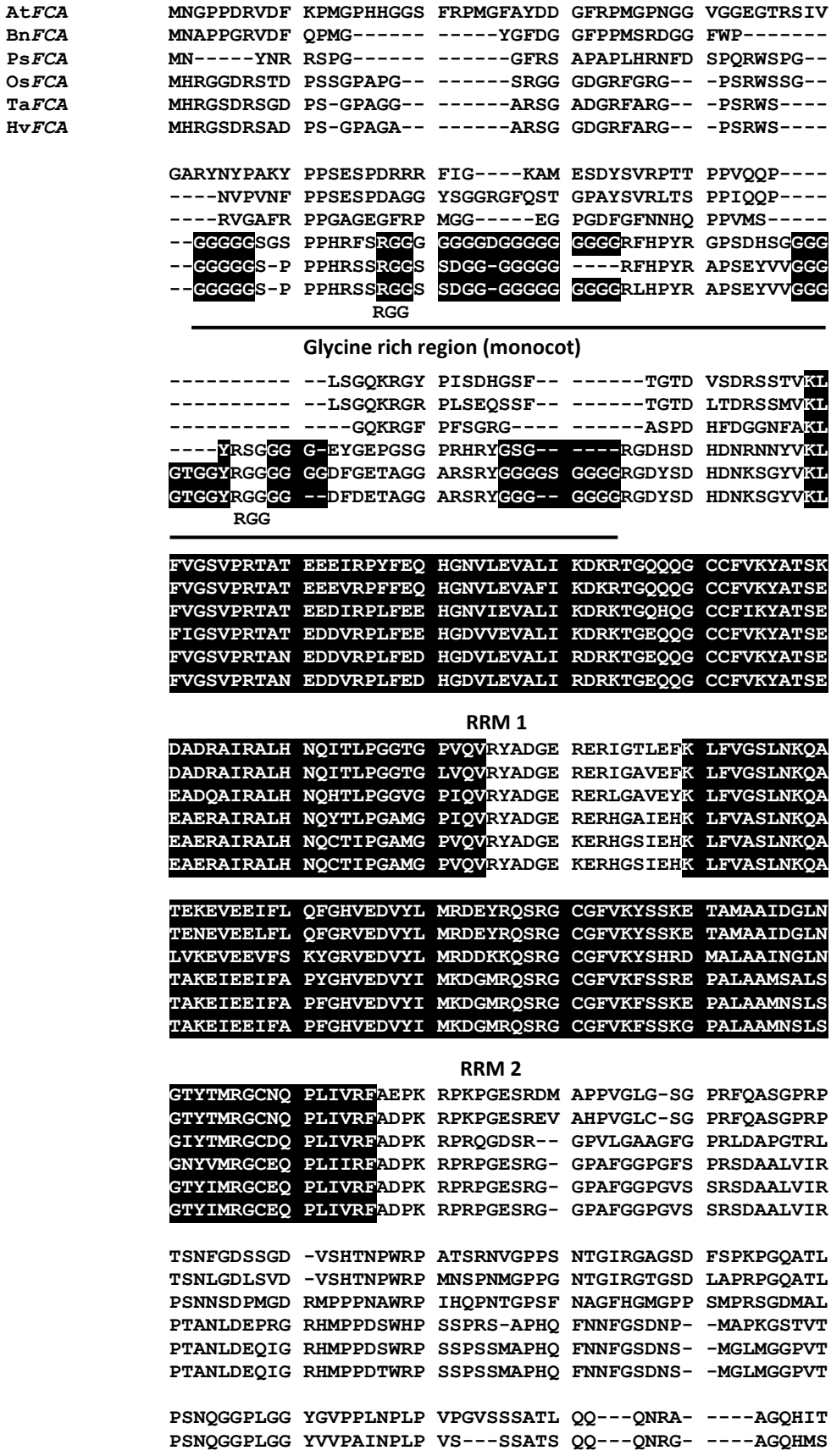
4.2 Sequence analysis of barley FCA

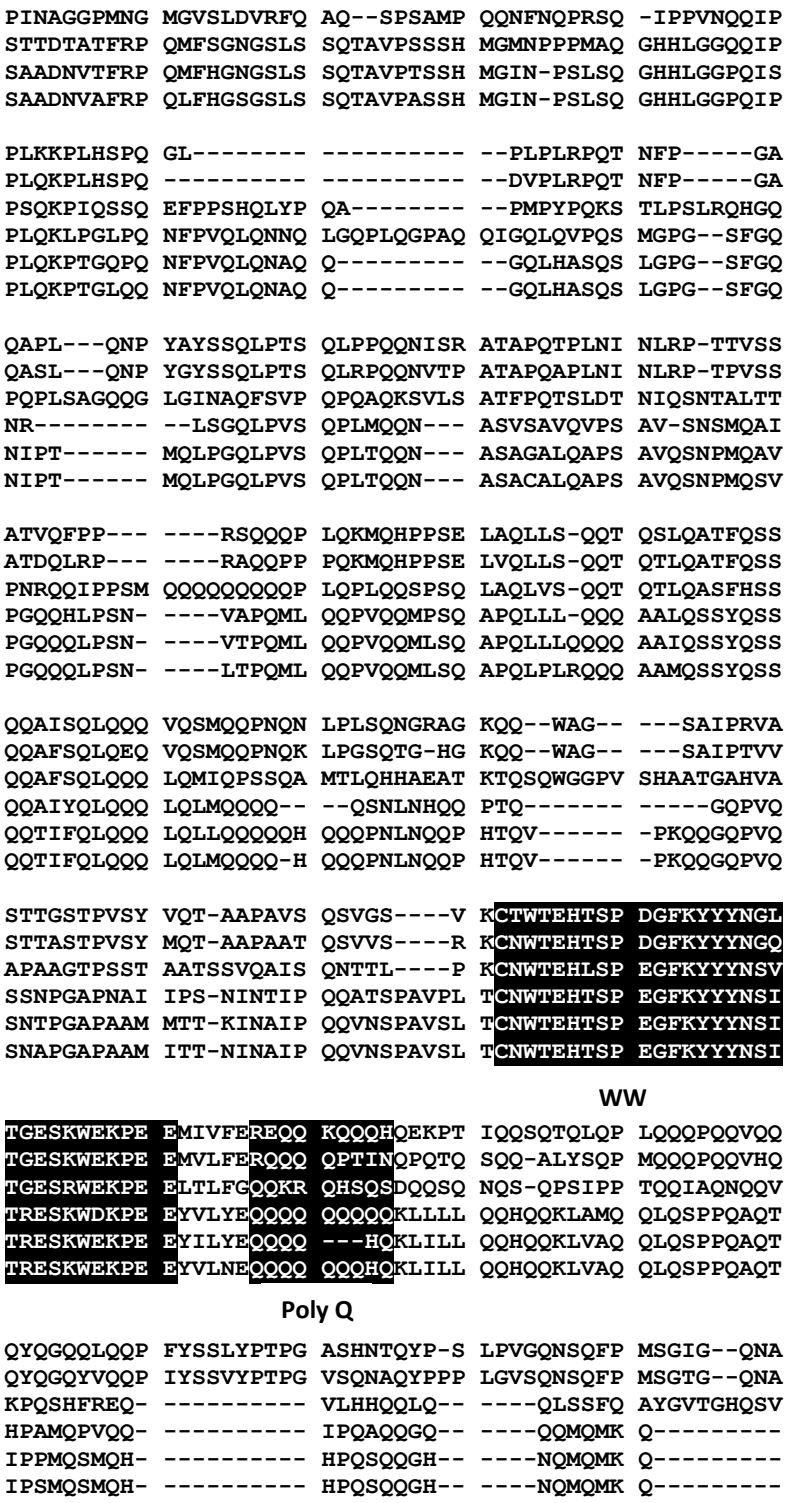
Comparing complete amino acid sequences, barley FCA showed higher sequence similarity to wheat (95%) and rice (79%) than to Arabidopsis (Z82989) (43%) (Fig. 4.6). The sequence similarity was very high (> 90%) when RRM and WW domains were compared in barley, wheat, rice and Arabidopsis. Interestingly, all monocot FCAs contained glycine-rich regions at the N-terminus of the protein corresponding to the GC rich region at the 5'end of the nucleic acid region which are absent from Arabidopsis FCA. Monocot FCA also contained RGG motifs in the form of single repeats and a longer polyglutamine (poly Q) region after the WW domain compared to Arabidopsis FCA (Fig. 4.6).

A phylogenic tree based on all known FCA protein sequences from monocots and dicots was generated (Fig. 4.7). The amino acid based phylogenic comparison showed clear divergence between monocot and dicot FCA. Among the monocots, barley FCA was more closely related to wheat than to rice FCA.

4.3 Characterization of barley *FCA* during seed maturation and germination

FCA is known to regulate the transition from vegetative to reproductive phase in plants and is well characterized in Arabidopsis during these phases of development (Macknight *et al.* 1997). Because *ABAP1* was initially described in caryopses, barley *FCA* was characterized during embryo maturation and germination.


AtFCA MNGPPDRVDF KPMGPHHGGS FRPMGFAYDD GFRPMGPNGG VGGEGTRSIV
BnFCA MNAPPGRVDF QPMG------ -----YGFDG GFPPMSRDGG FWP-------
PsFCA MN-----YNR RSPG------ ------GFRS APAPLHRNFD SPQRWSPG--
OsFCA MHRGGDRSTD PSSGPAPG-- ------SRGG GDGRFGRG-- -PSRWSSG--
TaFCA MHRGSDRSGD PS-GPAGG-- ------ARSG ADGRFARG-- -PSRWS----
HvFCA MHRGSDRSAD PS-GPAGA-- ------ARSG GDGRFARG-- -PSRWS----
GARYNYPAKY PPSESPDRRR FIG----KAM ESDYSVRPTT PPVQQP----
----NVPVNF PPSESPDAGG YSGGRGFQST GPAYSVRLTS PPIQQP----
----RVGAFR PPGAGEGFRP MGG-----EG PGDFGFNNHQ PPVMS-----
--GGGGGSGS PPHRFSRGGG GGGGDGGGGG GGGGRFHPYR GPSDHSGGGG
--GGGGGS-P PPHRSSRGGS SDGG-GGGGG ----RFHPYR APSEYVVGGG
--GGGGGS-P PPHRSSRGGS SDGG-GGGGG GGGGRLHPYR APSEYVVGGG
RGG
Glycine rich region (monocot)
---------- --LSGQKRGY PISDHGSF-- ------TGTD VSDRSSTVKL
---------- --LSGQKRGR PLSEQSSF-- ------TGTD LTDRSSMVKL
---------- ----GQKRGF PFSGRG---- ------ASPD HFDGGNFAKL
----YRSGGG G-EYGEPGSG PRHRYGSG-- ----RGDHSD HDNRNNYVKL
GTGGYRGGGG GGDFGETAGG ARSRYGGGGS GGGGRGDYSD HDNKSGYVKL
GTGGYRGGGG --DFDETAGG ARSRYGGG-- GGGGRGDYSD HDNKSGYVKL
RGG
FVGSVPRTAT EEEIRPYFEQ HGNVLEVALI KDKRTGQQQG CCFVKYATSK
FVGSVPRTAT EEEVRPFFEQ HGNVLEVAFI KDKRTGQQQG CCFVKYATSE
FVGSVPRTAT EEDIRPLFEE HGNVIEVALI KDRKTGQHQG CCFIKYATSE
FIGSVPRTAT EDDVRPLFEE HGDVVEVALI KDRKTGEQQG CCFVKYATSE
FVGSVPRTAN EDDVRPLFED HGDVLEVALI RDRKTGEQQG CCFVKYATSE
FVGSVPRTAN EDDVRPLFED HGDVLEVALI RDRKTGEQQG CCFVKYATSE
RRM 1
DADRAIRALH NQITLPGGTG PVQVRYADGE RERIGTLEFK LFVGSLNKQA
DADRAIRALH NQITLPGGTG LVQVRYADGE RERIGAVEFK LFVGSLNKQA
EADQAIRALH NQHTLPGGVG PIQVRYADGE RERLGAVEYK LFVGSLNKQA
EAERAIRALH NQYTLPGAMG PIQVRYADGE RERHGAIEHK LFVASLNKQA
EAERAIRALH NQCTIPGAMG PVQVRYADGE KERHGSIEHK LFVASLNKQA
EAERAIRALH NQCTIPGAMG PVQVRYADGE KERHGSIEHK LFVASLNKQA
TEKEVEEIFL QFGHVEDVYL MRDEYRQSRG CGFVKYSSKE TAMAAIDGLN
TENEVEELFL QFGRVEDVYL MRDEYRQSRG CGFVKYSSKE TAMAAIDGLN
LVKEVEEVFS KYGRVEDVYL MRDDKKQSRG CGFVKYSHRD MALAAINGLN
TAKEIEEIFA PYGHVEDVYI MKDGMRQSRG CGFVKFSSRE PALAAMSALS
TAKEIEEIFA PFGHVEDVYI MKDGMRQSRG CGFVKFSSKE PALAAMNSLS
TAKEIEEIFA PFGHVEDVYI MKDGMRQSRG CGFVKFSSKG PALAAMNSLS
RRM 2
GTYTMRGCNQ PLIVRFAEPK RPKPGESRDM APPVGLG-SG PRFQASGPRP
GTYTMRGCNQ PLIVRFADPK RPKPGESREV AHPVGLC-SG PRFQASGPRP
GIYTMRGCDQ PLIVRFADPK RPRQGDSR-- GPVLGAAGFG PRLDAPGTRL
GNYVMRGCEQ PLIIRFADPK RPRPGESRG- GPAFGGPGFS PRSDAALVIR
GTYIMRGCEQ PLIVRFADPK RPRPGESRG- GPAFGGPGVS SRSDAALVIR
GTYIMRGCEQ PLIVRFADPK RPRPGESRG- GPAFGGPGVS SRSDAALVIR
TSNFGDSSGD -VSHTNPWRP ATSRNVGPPS NTGIRGAGSD FSPKPGQATL
TSNLGDLSVD -VSHTNPWRP MNSPNMGPPG NTGIRGTGSD LAPRPGQATL
PSNNSDPMGD RMPPPNAWRP IHQPNTGPSF NAGFHGMGPP SMPRSGDMAL
PTANLDEPRG RHMPPDSWHP SSPRS-APHQ FNNFGSDNP- -MAPKGSTVT
PTANLDEQIG RHMPPDSWRP SSPSSMAPHQ FNNFGSDNS- -MGLMGGPVT
PTANLDEQIG RHMPPDTWRP SSPSSMAPHQ FNNFGSDNS- -MGLMGGPVT
PSNQGGPLGG YGVPPLNPLP VPGVSSSATL QQ---QNRA- ----AGQHIT
PSNQGGPLGG YVVPAINPLP VS---SSATS QQ---QNRG- ----AGQHMS


WW
Poly Q

```
QDYARTHIPV GAASMNDISR TQQSRQSPQE LMWKNKA---
QEFGRTDTPV GVASINNPSR TQQVHERKS- ----------
QELGYKQT-- SFVSAGGPGR YSQGINTAQE LMWKNRPAGV
QELNYTQL-- QTPGAIDPSR IQQGIQSAQE RAWKS-----
QDLNYNQL-- QATGTIDPSR IQQGIQAAQE RSWKS-----
QDLNYNQL-- QPTGTIDPSR IQQGIQAAQE RSWKS-----
```

Figure 4.6. Amino acid sequence comparison of various species of monocot and dicot FCA. The different conserved domains in FCA are represented by shaded regions with their names below. The region encompassing the glycine rich islands is shown by a straight line below the sequences. The RGG motifs are labelled below where they are present in the protein sequence.

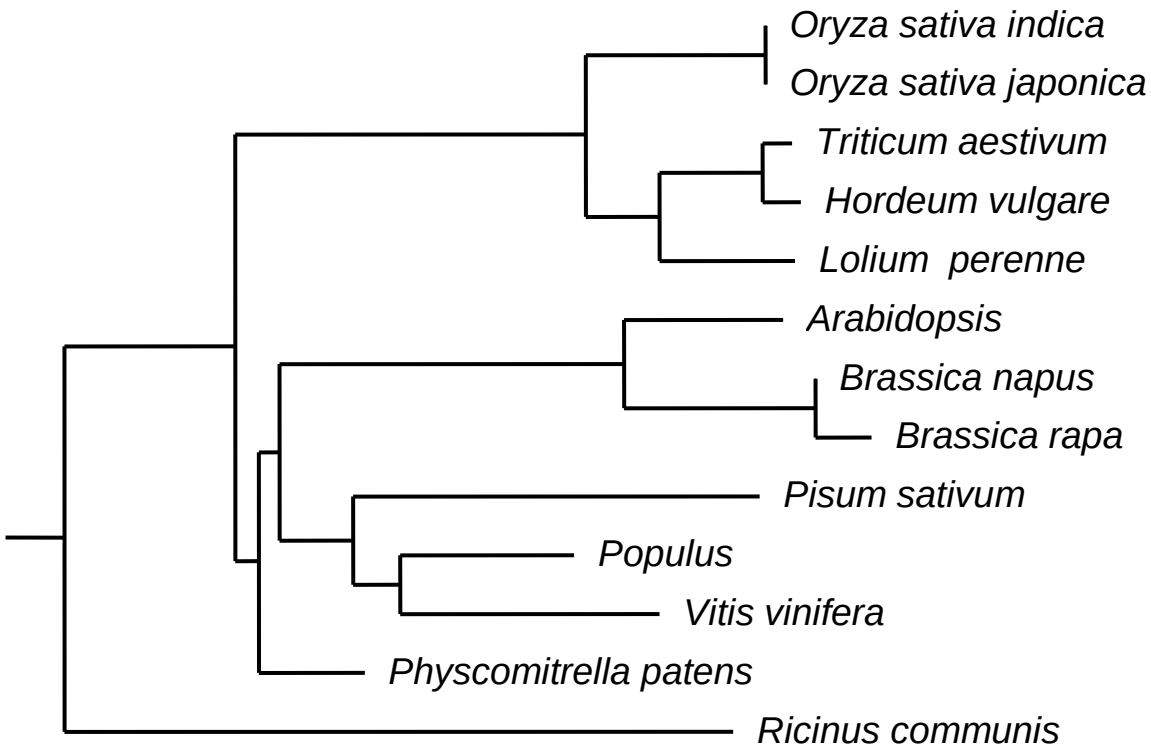


Figure 4.7. Phylogenic tree of FCA showing the evolutionary relationship between barley FCA and FCA proteins identified from other plant species. The proteins having highest percent sequence similarity are grouped together. See Table 1 for accession numbers and list of selected proteins.

Table 1: Protein sequences selected for phylogenetic analysis shown in **Figure 4.7**.

Serial No.	Name	Acc. No.	Description	Reference
1	FCA gamma	FJ188402	Flowering time control protein (*Hordeum vulgare*)	Current study
2	FCA gamma	AF414188	Flowering time control protein (*Brassica napus*)	(Macknight *et al.* 2002)
3	FCA	EU700363	Flowering time control protein (*Brassica rapa*)	Xiao *et al.* 2008 (Direct Submission)
4	FCA gamma	Z82989	RNA binding protein-protein interaction domain containing protein	(Macknight *et al.* 1997)
5	FCA gamma	AY805329	Flowering time control protein (*Pisum sativum*)	(Hecht *et al.* 2005)
6	FCA gamma	AY654584	Flowering time control protein (*Oriza sativa* (" japonica" cultivar-group)	Winnichayakul *et al.* 2004 (Direct Submission)
7	OsFCA-1	AY274928	Flowering time control protein (*Oriza sativa* (" indica" cultivar-group)	(Du *et al.* 2006)
8	FCA-A1	AY230398	Flowering time control protein (*Triticum aestivum*)	Gao et al. 2004 (Direct Submission)
9	FCA gamma	AY654582	Flowering time control protein (*Lolium perenne*)	Winnichayakul *et al.* 2004 (Direct Submission)
10	Predicted protein	XP_002316051	Hypothetical protein (*Populus trichocarpa*)	(Direct Submission)
11	Predicted protein	XP_001773118	Hypothetical protein (*Populus trichocarpa*)	(Direct Submission)
12	Predicted protein	XP_002279515	Hypothetical protein (*Vitis vinifera*)	(Direct Submission)
13	RNA binding protein	XP_002520700	RNA binding protein (*Ricinus communis*)	(Direct Submission)

During barley embryo development and maturation, *FCA* transcripts could be detected from 2 days after pollination (DAP) to late maturation or desiccated embryo stage (Fig. 4.8 A & B). Reverse-transcriptase PCR detected *FCA* transcripts as early as 2 DAP and the transcripts were present up to 21 DAP with no detectable variation (Fig. 4.8 A). *FCA* transcripts showed little variation over the course of embryo maturation, but band intensity analysis indicated that the *FCA* transcript levels were slightly higher in seeds 40 DAP compared to other studied stages (Fig. 4.8 B & Fig. 4.3).

FCA transcript changes during embryo germination were also determined. The embryos showed 93% germination by 24 hrs. after the state of imbibition and complete germination by the end of 30 hrs. after imbibition in water (Fig. 4.9 A). After 16 hours of imbibition, *FCA* transcripts declined by about 50% (Fig. 4.9 B). The *FCA* transcripts showed the greatest decline between 0 to 16 hrs., however, the rate of decline per hour for FCA transcripts was greatest between 16 and 24 hrs. After germination was complete, *FCA* transcript levels were relatively constant (Fig. 4.9 B). To determine if *FCA* expression is affected by imbibition in ABA and by the state of germination, embryos were imbibed with 10µM ABA and germination followed for 48 hrs. Embryos imbibed in ABA showed reduced germination during 48 hrs. (Fig. 4.10 A). The embryos did not show any signs of germination for the first 16 hrs. and showed only 8% germination in the first 24 hrs. After 48 hrs, germination reached only 40% (Fig. 4.10 A). Samples of embryos treated with ABA were collected at 24 hrs. and 48 hrs. to determine the transcript levels of *FCA*.

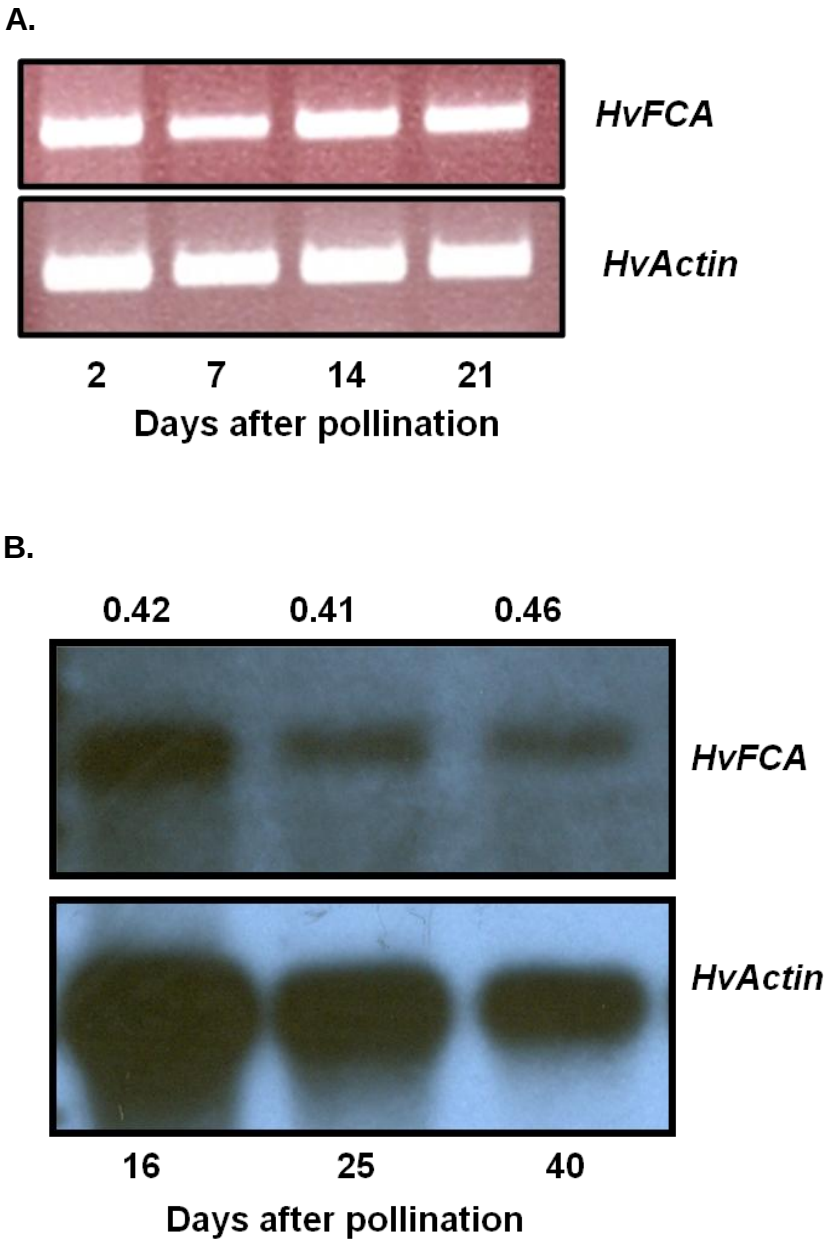


Figure 4.8. *FCA* expression pattern during barley embryo development. The figures in (A) and (B) represent results from different sets of RNA. **(A)** RT-PCR analysis of *FCA* transcript abundance. The lane numbers represent days after pollination (DAP). **(B)** Northern blot analysis of *FCA* transcript abundance. The lane numbers represent days after pollination (DAP). The values above the autoradiogram depict gene/actin band intensity ratios.

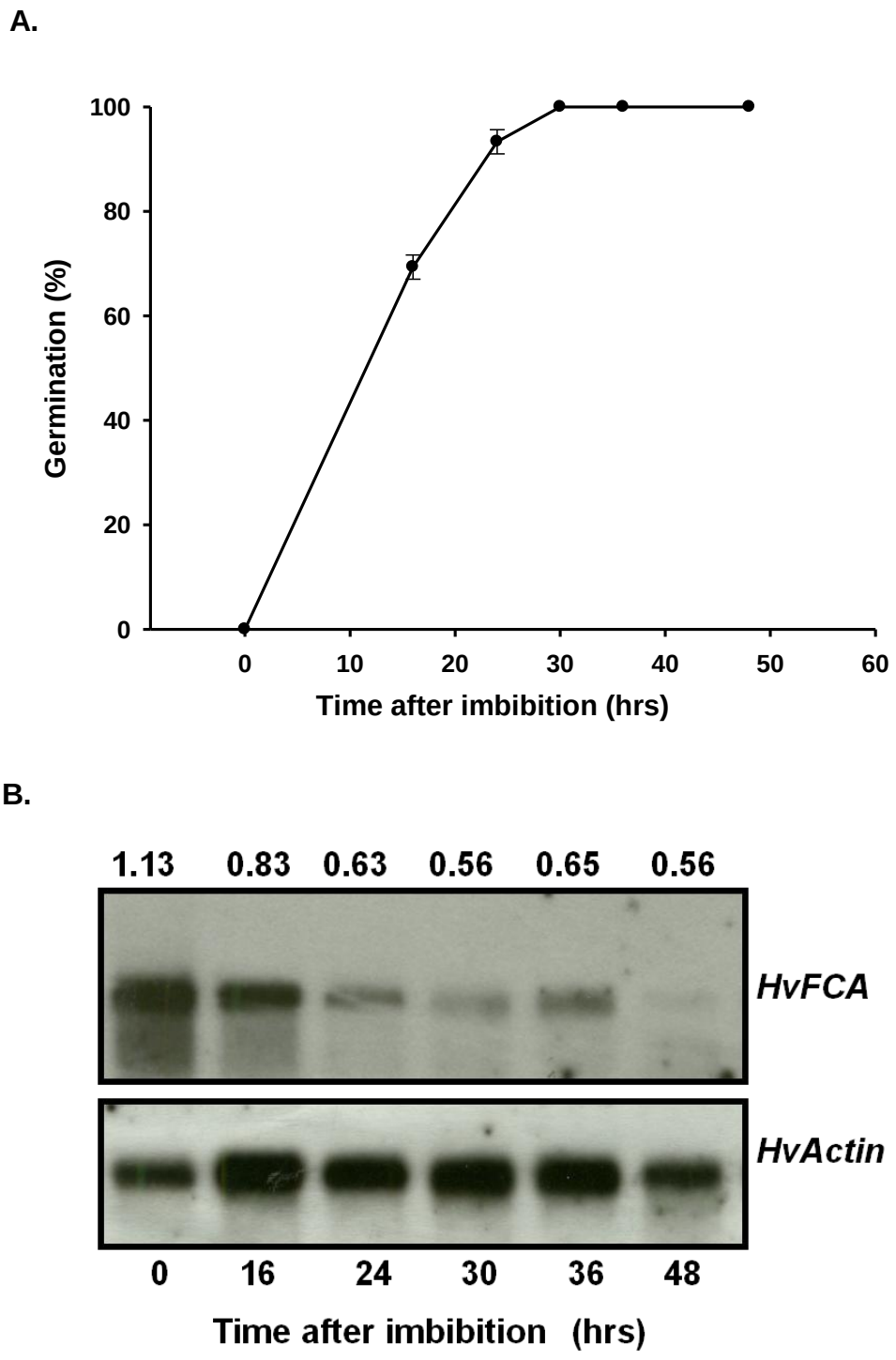


Figure 4.9. Germination and *FCA* transcript profile in cultivar McLeod barley (2004 harvest) embryos in water. **(A)** Germination of excised barley embryos. The error bars represent standard deviation values from three replicates. **(B)** Northern blot analysis of *FCA* transcripts during germination of barley embryos. The lane numbers represent time in hours. The values above the autoradiogram depict gene/actin band intensity ratios.

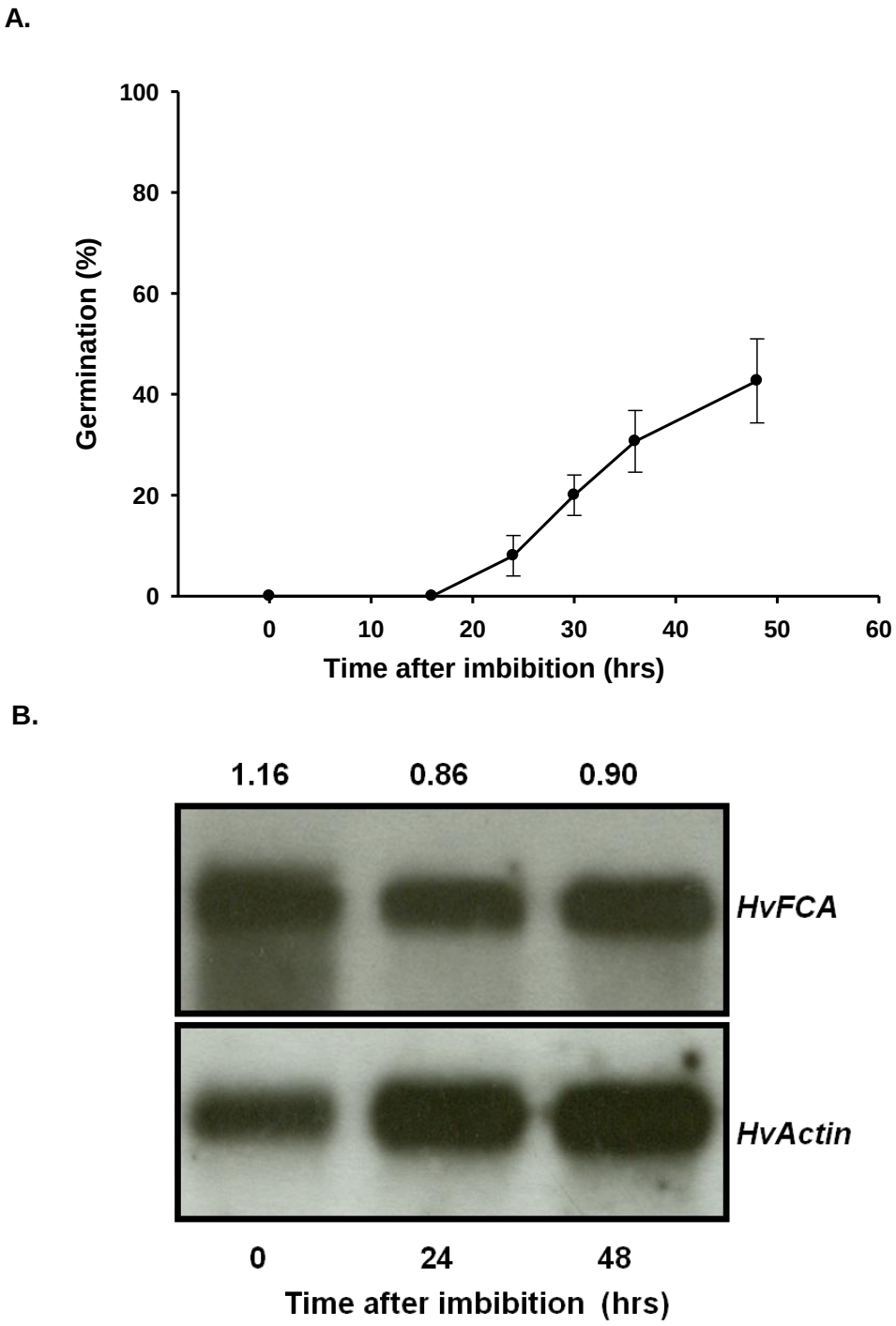


Figure 4.10. Germination and transcript profile of *FCA* in excised barley (2004 harvest) embryos in the presence of 10 µM ABA. **(A)** Germination of excised barley embryos over time in the presence of ABA. Error bars represent the standard deviation values from three replicates. **(B)** Northern blot analysis of *FCA* transcripts during germination of barley embryos over time in the presence of ABA. The lane numbers represent time in hours. The values above the autoradiograms depict gene/actin band intensity ratios.

FCA levels in embryos treated with ABA declined about 25% in the first 24 hrs. but then remained stable between 24 to 48 hrs. After the state of imbibition (Fig. 4.10 B).

4.4 *FCA* response to ABA and salt stress

To determine whether ABA could up-regulate *FCA* transcripts once it had declined during embryo germination, embryos were imbibed in water for 24 hrs. and then incubated in concentrations of ABA ranging from 0 µM to 20 µM ABA for 24 hrs. *FCA* transcripts increased in 24 hrs-imbibed embryos with ABA treatment compared to the embryos that were treated with water for 48 hrs. (Fig. 4.11 A). Although there were higher levels of *FCA* transcripts in germinated embryos after 24 hrs. of treatment with different ABA concentrations, there was not a dose-dependent response of *FCA* transcripts to applied ABA. *FCA* transcripts increased 1.6, 1.7, 1.3 and 1.54 fold under 2, 5, 10 and 20 µM ABA treatments respectively compared to untreated embryos (Fig. 4.11 A). To test if *FCA* transcripts were up-regulated by salt stress, embryos were imbibed in water for 24 hrs., then treated with two concentrations of NaCl. Salt treatments of 100 and 200 mM NaCl increased *FCA* transcripts by 1.16 and 1.41 fold respectively compared to the water-treated control (Fig. 4.11 B).

FCA response to ABA and salt stress was also studied in barley seedlings (first green leaf stage). Five day old barley seedlings were treated with 20 µM ABA or 400 mM salt (NaCl) in half-strength MS medium for 24 hrs. and analysed for changes in *FCA* expression. *FCA* transcripts were up-regulated by ABA and

salt treatment, whereas in untreated seedlings *FCA* transcripts could not be detected (Fig. 4.11 C).

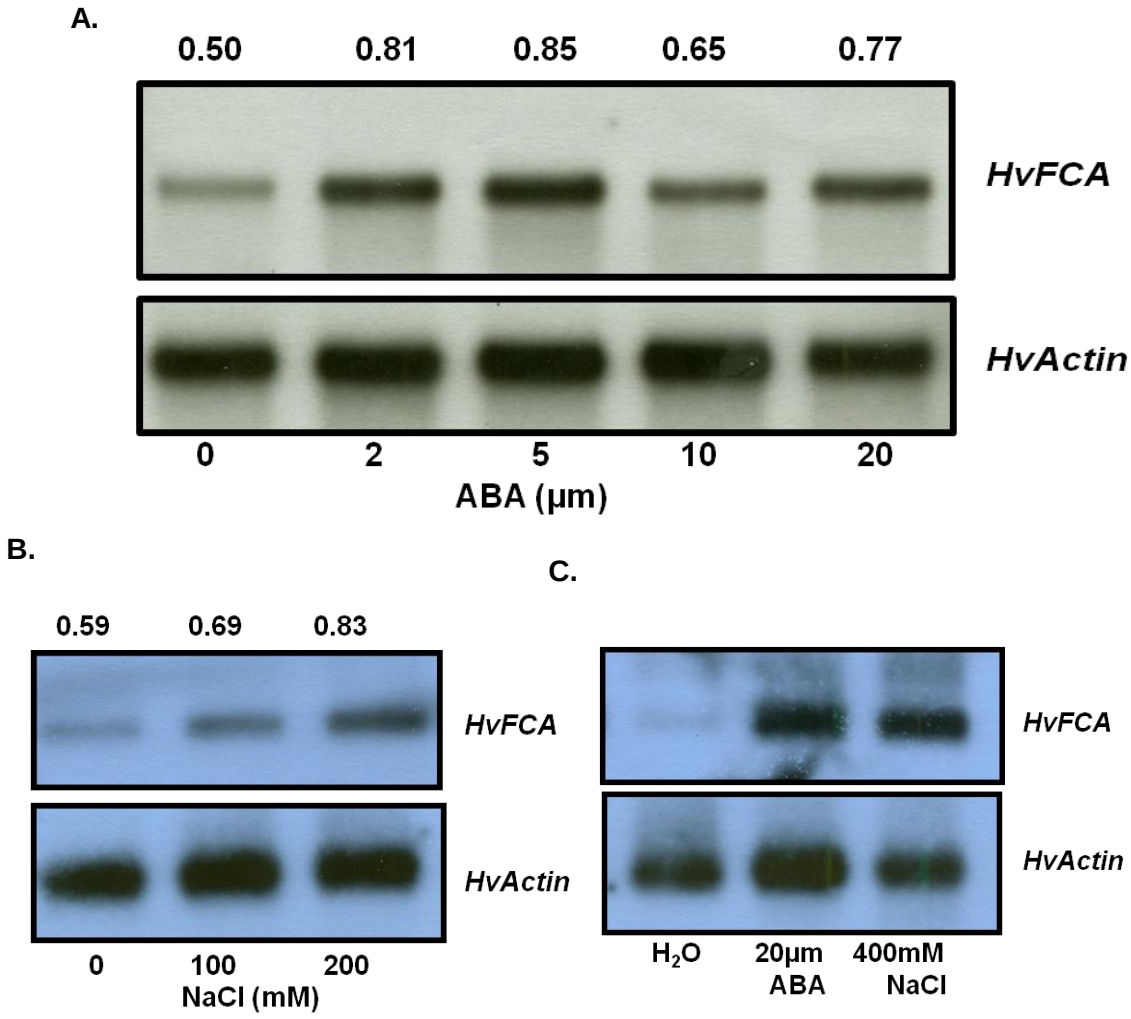


Figure 4.11. Analysis of *FCA* transcripts in germinated barley (2007 harvest) embryos by northen blotting. **(A)** Northern Blot analysis of *FCA* transcript levels in barley embryos germinated in water for 24 hrs. before treatment with increasing concentration of ABA. The lane numbers represent concentration of ABA (µM). The values above the autoradiograms depict gene/actin band intensity ratios. **(B)** Northern Blot analysis of *FCA* transcript levels in barley embryos germinated in water for 24 hrs.before treatment with increasing concentration of salt (NaCl). The lane numbers represent concentration of NaCl (mM). **(C)** Northern Blot analysis of *FCA* transcript levels in 5 day old barley seedlings treated with ½ MS medium (C) or ½ MS medium containing ABA or NaCl.

4.5 Barley *FCA* effect on ABA responsive promoters

Experiments were conducted to see if FCA could affect promoters of genes that have been shown to respond to ABA. Em is a cereal late embryogenesis protein involved in desiccation tolerance that is up-regulated by ABA (Marcotte *et al.* 1988), while VP1 is a transcription factor involved in dormancy and also up-regulated by ABA (Robichaud and Sussex 1986). Promoter-reporter constructs of the two genes were used in transient expression experiments to determine the effect of *FCA* on their expression. Barley *FCA* gene was co-expressed with either P_{Em}-*GUS* or P_{VP1}-*GUS* along with a P_{35S}-*LUC* (used as transformation control) construct to study the effect of *FCA* on the expression of *Em* and *VP1*.

Increased GUS activity was observed when *FCA* was co-expressed with P_{Em}-*GUS* in either the presence or absence of ABA (Fig. 4.12). Anti-sensing or knockdown of *FCA* decreased GUS activity from P_{Em}-*GUS* either in the absence or presence of ABA but the differences with respect to the empty vector control were not significant (Fig. 4.12). The induction of GUS activity due to ABA treatment alone was 8.9 fold, significantly higher than no-ABA-treated empty vector transformed conditions. The co-expression of *FCA* led to significantly higher GUS activity in the absence (3.6 fold) or presence (3.2 fold) of ABA compared to empty vector control.

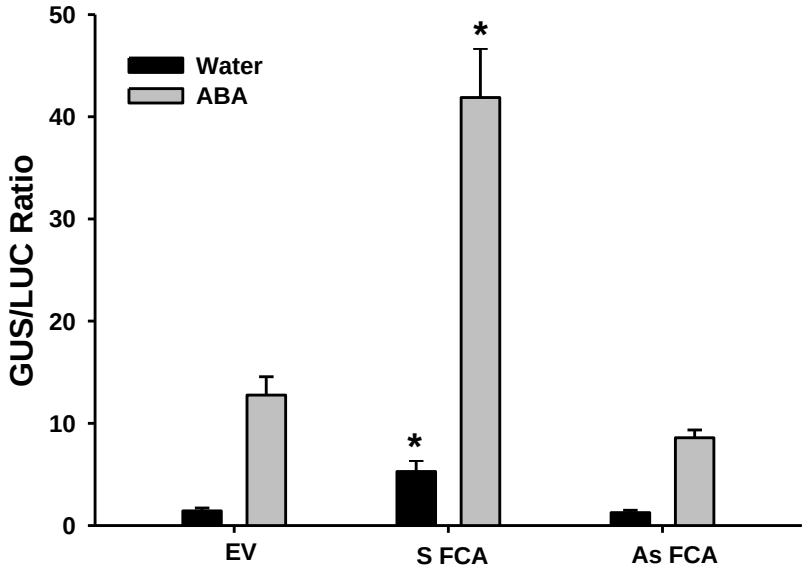


Figure 4.12. Effect of empty vector (EV), sense *FCA* (S *FCA*), and anti-sense *FCA* (As *FCA*) on expression of barley *Em* gene promoter tagged to β-glucuronidase (GUS) reporter in barley aleurone protoplasts treated with water or 10 µM ABA for 48 hrs. The error bars represent standard deviation values from three replicates.

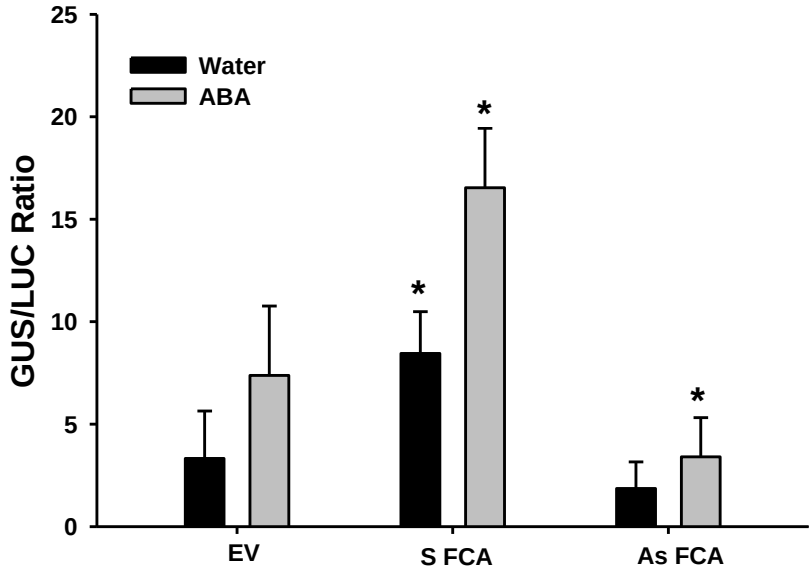


Figure 4.13. Effect of empty vector (EV), sense *FCA* (S *FCA*), and anti-sense *FCA* (As *FCA*) on expression of maize *VP1* gene promoter tagged to β-glucuronidase (GUS) reporter in barley aleurone protoplasts treated with water or 10 µM ABA for 48 hrs. The error bars represent standard deviation values from three replicates.

Transient co-expression of *FCA* with P_{VP1}-*GUS* also resulted in increased measurable GUS activity in either the presence or absence of ABA (Fig. 4.13). There was a significant increase in GUS activity due to co-expression of *FCA* in the absence (2.5 fold) or presence (2.3 fold) of ABA compared to empty vector control. Anti-sensing of *FCA* caused significant decline in GUS activity under ABA-treated (2.1 fold) but not under water-treated conditions (1.8 fold).

FCA protein contains two well-characterized domains, an RRM which interacts with nucleic acids (Liu *et al.* 2007a) and a WW domain for interaction with other proteins (Quesada *et al.* 2003). Since *FCA* affects *Em* and *VP1* expression, an investigation was performed to determine whether the RRM domain of FCA was required for this effect. *FCA* contains an ATG sequence, required for initiation of translation, at the end of the second RRM (exon 8) which maintains the correct open reading frame of *FCA* (earlier called as ABAP1; Appendix 3). The fragment of *FCA* starting from ATG in exon 8 to the stop codon was cloned and used as truncated *FCA* lacking the RRM (ABAP1). The effect of truncated *FCA* (*ABAP1*) and *FCA* on the two promoters were carried out simultaneously.

Co-transient expression of truncated *FCA* with P_{Em}-*GUS* in barley aleurone protoplasts resulted in significantly higher GUS activity compared with an empty vector control, in the presence (3.1 fold) or absence of ABA (2.9 fold) (Fig. 4.14).

Transient co-expression of truncated *FCA* with P_{VP1}-*GUS* also resulted in increased measurable GUS activity in either the presence (2.2 fold) or absence (2.1 fold) of ABA (Fig. 4.15). This increase was not significant for the untreated samples, but in ABA-treated samples, the increase in GUS activity was significant (Fig. 4.15).

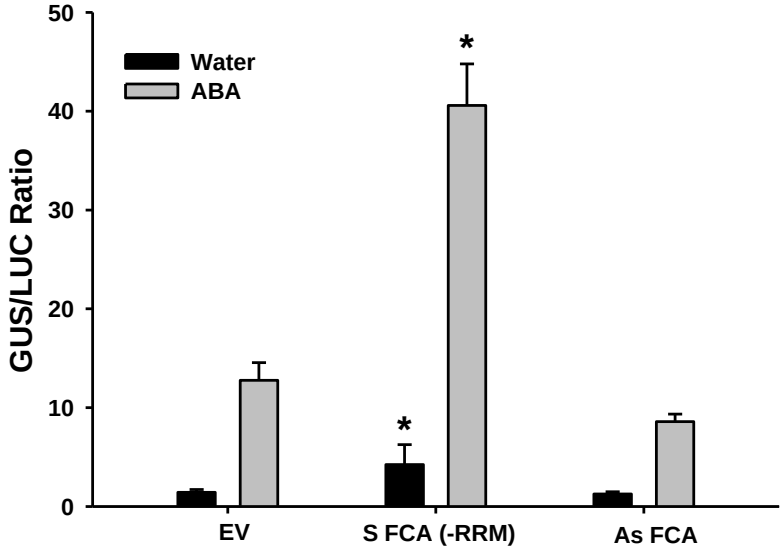


Figure 4.14. Effect of empty vector (EV), sense *FCA* without RRM (-RRM) , and anti-sense *FCA* (As *FCA*) on expression of barley *Em* gene promoter tagged to β-glucuronidase (GUS) reporter in barley aleurone protoplasts treated with water or 10 µM ABA for 48 hrs. The error bars represent standard deviation values from three replicates.

Anti-sensing of *FCA* did not cause a significant decline in GUS activity in untreated samples (1.8 fold), but the decline in GUS activity in ABA-treated embryos was significant (2.1 fold).

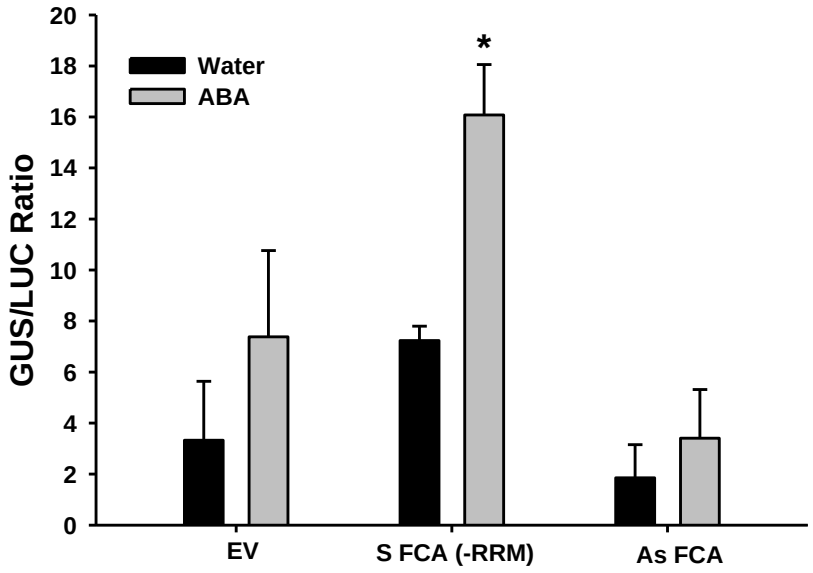


Figure 4.15. Effect of empty vector (EV), sense *FCA* without RRM (-RRM), and anti-sense *FCA* (As *FCA*) on expression of maize *VP1* gene promoter tagged to β-glucuronidase (GUS) reporter in barley aleurone protoplasts treated with water or 10 µM ABA for 48 hrs. The error bars represent standard deviation values from three replicates.

4.6 Effect of *FCA* on genes involved in regulation of dormancy

In section 4.3 of this thesis it was determined that during germination, *FCA* transcripts declined after imbibition in water (Fig. 4.9). *FCA* also affected promoters of genes responsive to ABA (Fig. 4.12 & 4.13). This raised the question as to whether *FCA* might affect genes involved in the regulation of dormancy. *FCA*, in sense and antisense orientations, was transiently expressed in excised barley embryos by particle bombardment of the scutellar surface, and the effect of this treatment on the expression of *VP1*, *ABI5*, and *SLN1* was examined.

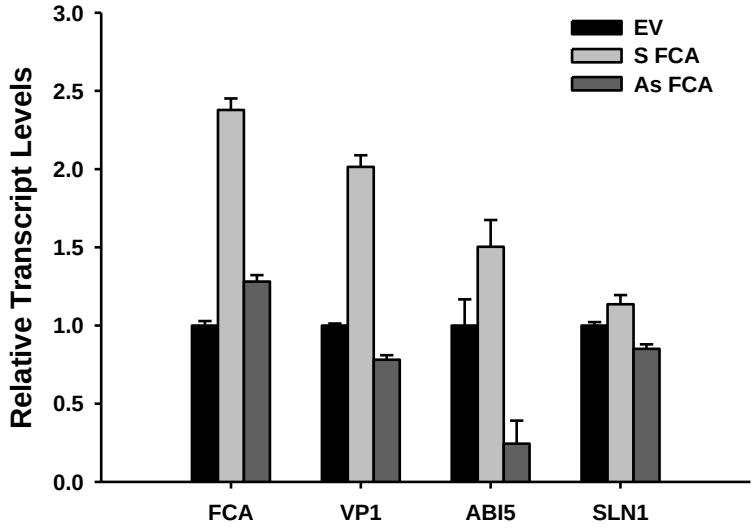


Figure 4.16. Effect of empty vector (EV), sense *FCA* (S *FCA*), and anti-sense *FCA* (As *FCA*) on expression of *VP1, ABAI5* and *SLN1* genes in excised barley (2007 harvest) embryos treated with water for 24 hrs. The values are represented as relative transcript levels compared to empty vector control analysed through real time PCR. The error bars represent standard deviation values from three replicates.

Bombardment with sense *FCA* led to significant increase (2.4 fold) in embryo *FCA* relative to the empty vector control bombardment, whereas bombardment with antisense *FCA* produced a slight increase (Fig. 4.16). There was a 2 fold significant increase in *VP1*, while bombarding with anti-sense *FCA* resulted in a 20% decline. The transcript levels of *ABI5* increased due to over-expression of *FCA* and decreased when *FCA* was anti-sensed but the differences were not significant (Fig. 4.16). The levels of *SLN1* showed no significant change when *FCA* was over- or under-expressed, although the changes followed the same trends as the other transcripts measured (Fig. 4.16).

In ABA-treated samples, no significant changes in transcript levels of *FCA*, *VP1* and *ABI5* were observed by over- or under-expressing *FCA*; however, *SLN1* transcripts increased significantly due to over-expression of *FCA* compared to empty vector control (Fig. 4.17).

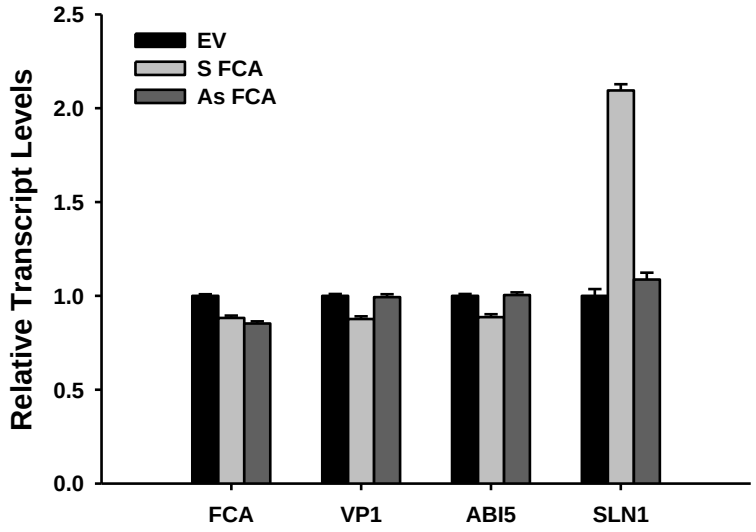


Figure 4.17. Effect of empty vector (EV), sense *FCA* (S *FCA*), and anti-sense *FCA* (As *FCA*) on expression of *VP1, ABI5* and *SLN1* genes in excised barley (2007 harvest) embryos treated with 10µM ABA for 24 hrs. The values are represented as relative transcript levels compared to empty vector control analysed through real time PCR. The error bars represent standard deviation values from three replicates.

4.7 Transmission of signal from scutellum to axis

Transient co-expression studies using reporter (tagged to GUS or LUC) and effector (gene of interest) constructs have been used to study the effect of genes on downstream signalling components (Lanahan *et al.* 1992). Since only a small proportion of cells are transformed by particle bombardment (Knudsen and

Muller 1991), it was surprising that one could see large-scale changes in transcripts of *VP1* when bombarded with *FCA* in the embryo (Fig. 4.16). To determine the extent of penetration of the particles during bombardment, a construct expressing GUS under the 35S constitutive promoter (pBI121 vector) was used to bombard the scutellar surface and after 24 hrs. the scutellum was dissected from the embryonic axis. GUS activity was then measured in the scutellum and embryonic axis using the MUG assay. The bombarded scutellum had 2.5 fold higher activity compared to the non-bombarded control (Fig. 4.18). In the embryonic axis, although there was a slight increase in GUS activity between the bombarded and non-bombarded samples, the difference was not significant. The GUS activity levels were comparable to the scutellum non-bombarded control. The background level of activity in the non-bombarded control is likely due to the presence of endogenous GUS in the embryos (Hu *et al.* 1990).

To determine whether bombardment of the scutellum with *FCA* could affect events in the embryonic axis, the scutellar surface of barley embryos was bombarded with *FCA* and the embryos incubated for 24 hrs. before dissecting the scutellum from the embryonic axis. As a result of the bombardment, transcript levels of *FCA* in the scutellum increased significantly by 5-fold compared to empty vector control and the transcript level of *VP1* increased significantly by 3-fold (Fig. 4.19 A). In the embryonic axis, there was no significant increase in levels of *FCA* transcript between the *FCA* bombarded samples compared to empty vector control (Fig. 4.19 B) while the transcript levels of *VP1* increased significantly by 50% compared to an empty vector control.

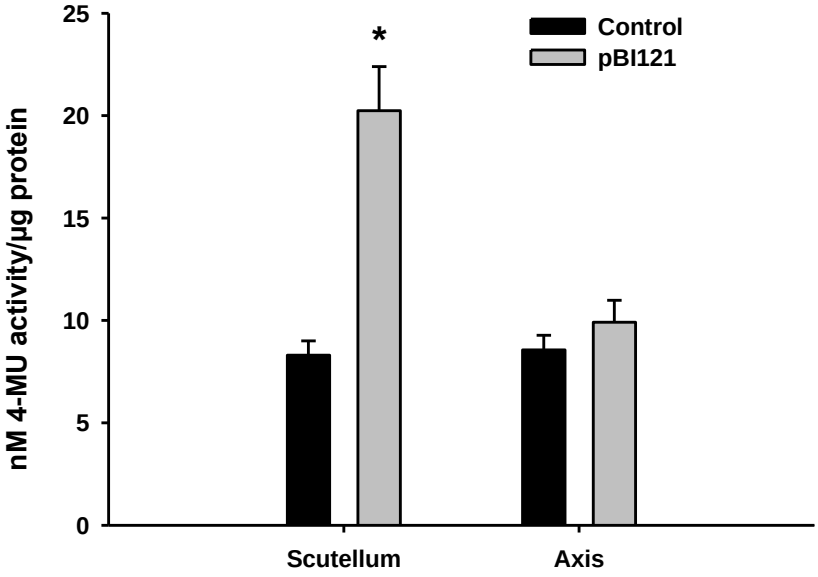
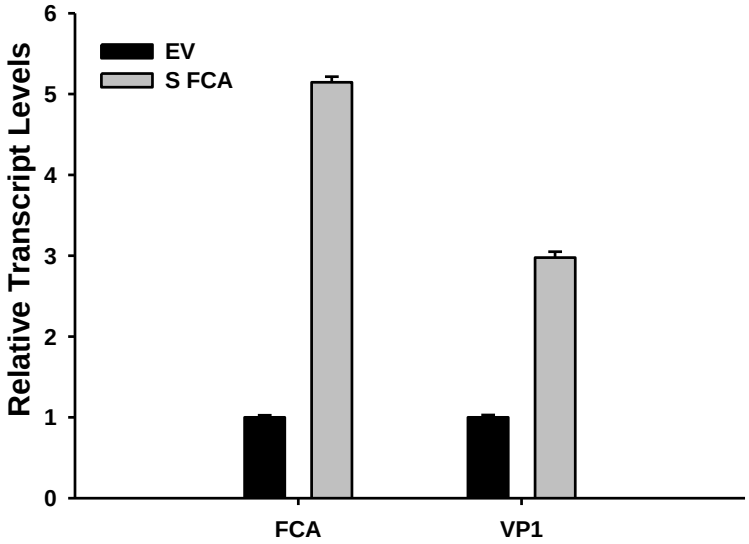


Figure 4.18. GUS activity in scutellum and embryonic axis tissue. The scutellar surface was transformed with a 35S promoter-driven GUS construct (pBI121). The embryo axis was separated after 24 hrs. of incubation following transformation and analysed separately. The error bars represent standard deviation values from three replicates.

A.



B.

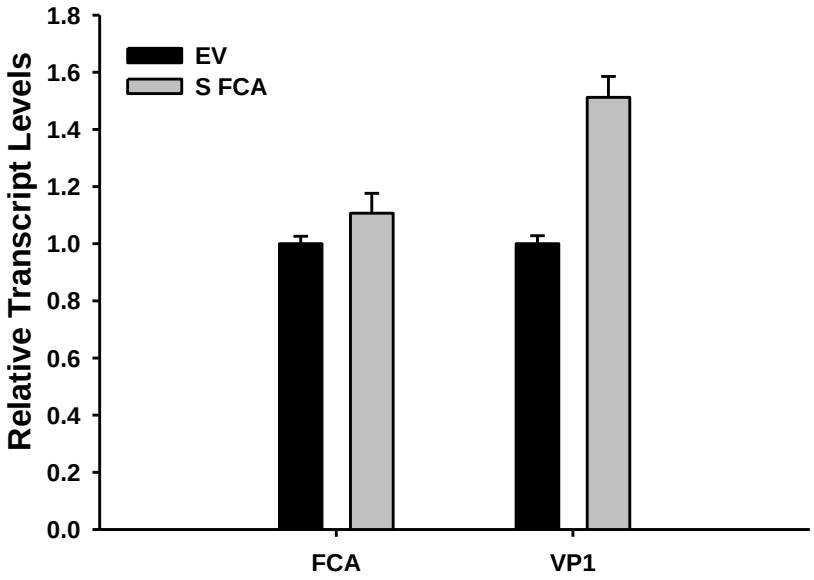


Figure 4.19. Effect of empty vector (EV) and sense *FCA* (S *FCA*) transformation of scutellar surface and its resulting effect on expression of *FCA* and *VP1* gene in **(A)** scutellum and **(B)** embryonic axis. The embryonic axis was separated after 24 hrs. of incubation following transformation and analysed separately. The values are represented as relative transcript levels compared to empty vector control. The error bars represent standard deviation values from three replicates.

4.8 Sub-cellular localization of barley *FCA*

Consistent with the functionality of an RNA binding protein, Arabidopsis FCA localizes in the nucleus in onion epidermal cells (Quesada *et al.* 2003). Analysis of the barley FCA protein sequence using the protein localization software “Predict NLS online” indicated the presence of a nuclear localization signal, KRPRP, at position 295 of the amino acid sequence. Analysis of the

Arabidopsis homolog sequence (Z82989) with the same software did not show the presence of any nuclear signal.

To determine localization within the cell, a barley *FCA* construct was prepared to yield an FCA-yellow fluorescent fusion (FCA-YFP) protein. This construct was transiently expressed in onion epidermal cells. Barley FCA-YFP fusion protein localized to the nucleus (Fig. 4.20 A & B).

To determine whether the RRM motifs are required for nuclear localization, the truncated $FCA_{(-RRM)}$ (without RRM), used in transient gene expression studies, was tagged in translational fusion to a red florescence protein (RFP) under control of the cauliflower mosaic virus promoter (*35S*) and *nos* terminator. Since it is easy to get confused between the nucleus and the protein storage vacuoles in the mature aleurone layer protoplasts, full length *VP1* gene in translational fusion to green fluorescent protein (GFP) that localized in nucleus of barley aleurone layers (Marella and Quatrano 2007), was used for identification of the nucleus. Protoplasts were also transformed with truncated $FCA_{(-RRM)}$-RFP alone as well as with VP1-GFP construct by PEG and $CaCl_2$ mediated transformation (Bruce *et al.* 1989). The transformed protoplasts were visualized 18-22 hrs. after transformation. Since auto-fluorescence is always a problem in aleurone protoplasts, the microscope was adjusted to the minimum laser intensity where the transformed cells had minimum fluorescence and the non-transformed cells had no fluorescence. Confocal imaging of aleurone protoplasts transformed without the reporter plasmids showed no fluorescence (Fig. 4.21 A & B). The protoplasts co-transformed with both truncated $FCA_{(-RRM)}$-RFP and VP1-GFP

showed that VP1 was localized in the nucleus (Fig. 4.21 C & D). The same cell that showed nuclear localization of VP1 was also visualised for localization of $FCA_{(-RRM)}$-RFP using a different filter in the microscope that allowed only red signal to pass. $FCA_{(-RRM)}$-RFP was localized primarily in the nucleus (Fig. 4.21 E & F). The red signal was also found to be coming from some other smaller organelles (Fig. 4.21 F & H). The localization of $FCA_{(-RRM)}$ in the nucleus of aleurone protoplasts was also confirmed in cells that were transformed with $FCA_{(-RRM)}$-RFP alone (Fig 4.21 G & H).

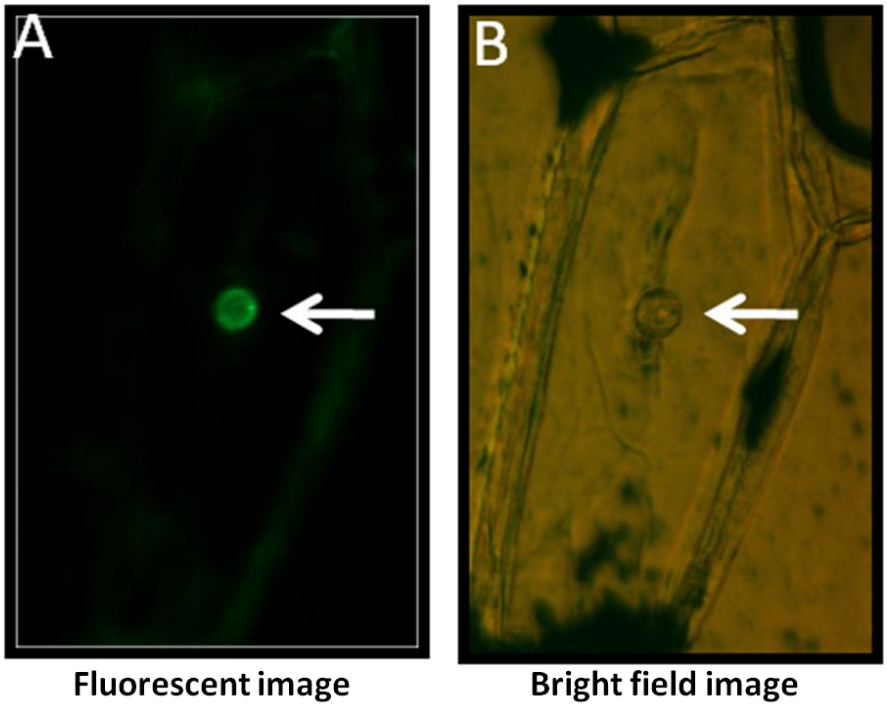


Figure 4.20. Localization of barley FCA-YFP in onion epidermal cells. **(A)** Fluorescent image of barley FCA-YFP signal from the nucleus. **(B)** Bright field image of onion epidermal cell showing the nucleus corresponding to Figure 4.20 A.

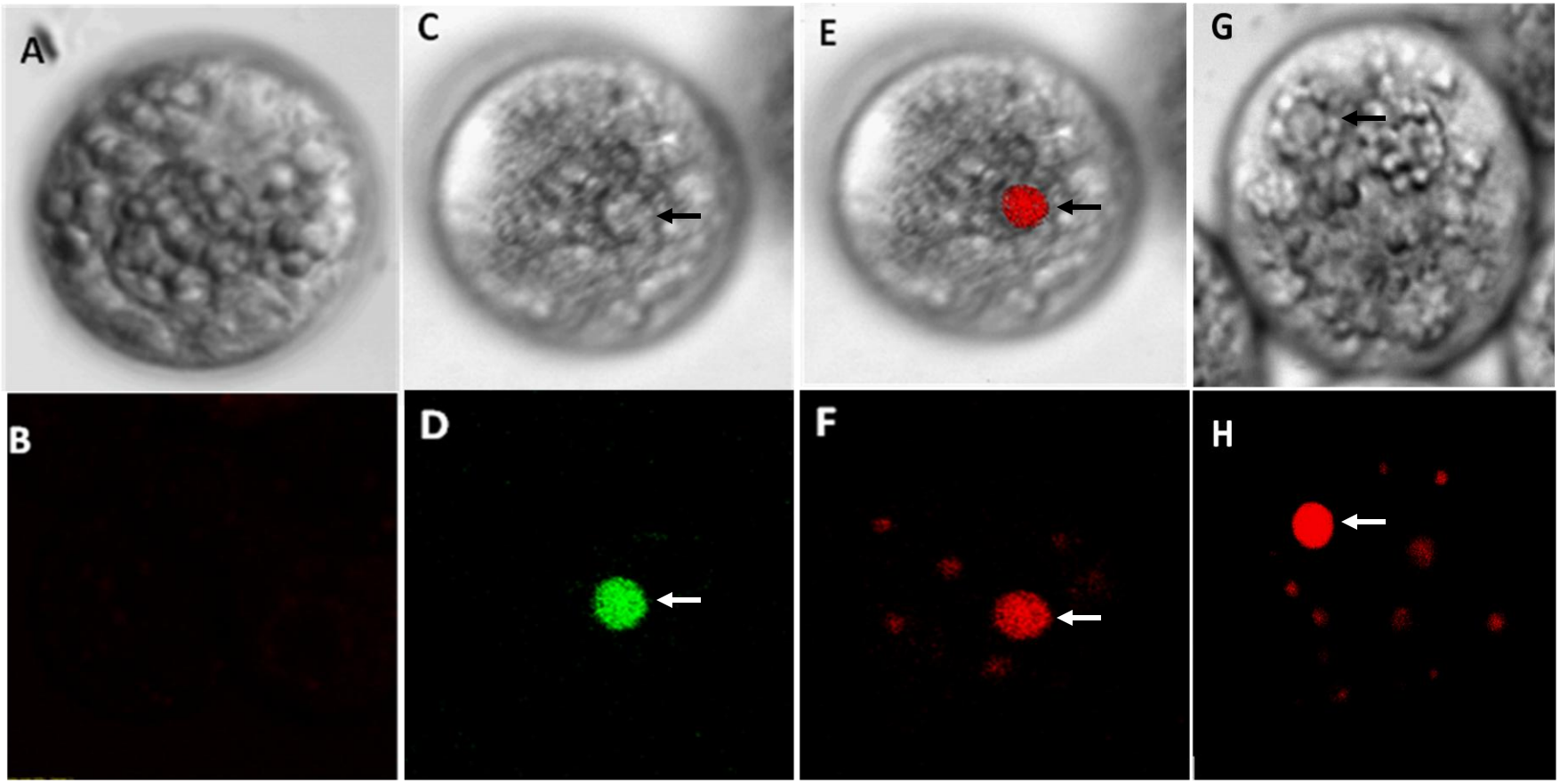


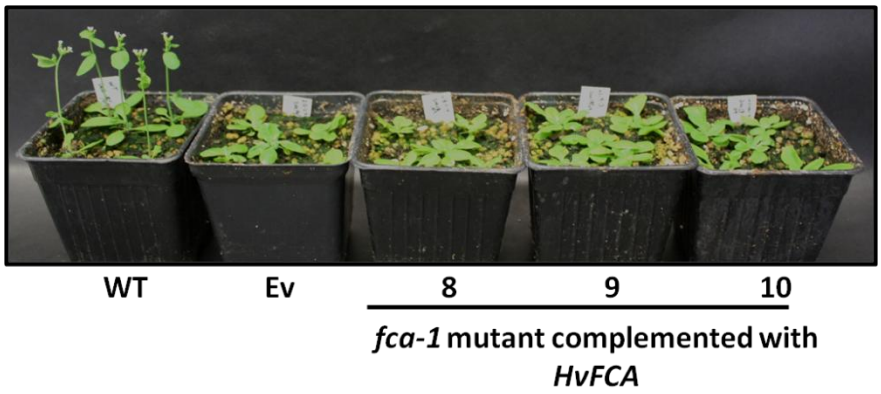
Figure 4.21. Localization of HvFCA$_{(-RRM)}$-RFP in barley aleurone protoplasts visualised with confocal microscopy. Images A, C, E and G represent the DIC image whereas images B, D, F & H were taken under red and green filters. A & B. Untransformed cells with no background signal. C,D, E & F are images from the same cell transformed with HvFCA$_{(-RRM)}$-RFP and VP-GFP (used as nuclear localization control). G and H are cells transformed with HvFCA$_{(-RRM)}$-RFP alone.

4.9 Barley *FCA* does not rescue the late flowering phenotype of Arabidopsis *fca-1* mutant

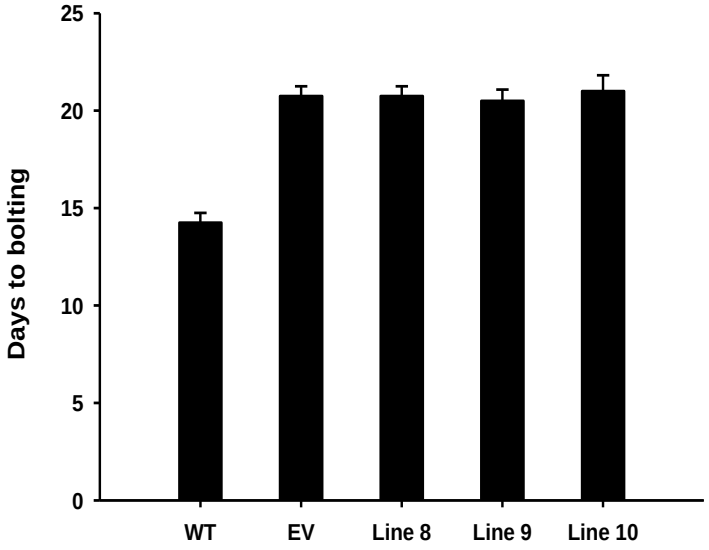
FCA affects flowering in Arabidopsis through regulation of FLC (Quesada *et al.* 2003). The rice homologue of FCA causes partial recovery of the late flowering phenotype of Arabidopsis *fca-1* mutant (Lee *et al.* 2005).

To further understand the properties of barley *FCA*, the Arabidopsis mutant *fca-1* was transformed with the barley gene to determine whether the late flowering phenotype could be rescued. The *fca-1* mutant complemented with barley *FCA* retained the late flowering phenotype of the mutant (Fig. 4.22 A). The time to bolting in wild type plants was less than 15 days, whereas the complemented lines and the mutant line took 21-23 days to bolt (Fig. 4.22 B). Northern blot analysis showed expression of barley *FCA* in the complimented lines when probed with barley *FCA* probe, whereas in the wild type and empty vector transformed lines the probe did not detect barley *FCA* (Fig. 4.22 C). The leaf number at flowering in wild type plants was lower compared to the complemented lines expressing empty vector or barley *FCA* (Fig. 4.22 D). The wild type plants had on average 8 leaves while the complemented lines had 13-14 leaves. Finally, *FLC* transcript levels were analysed in wild-type and complemented lines. The results demonstrated that *FLC* transcripts were present in *fca-1* mutant lines that were either expressing empty vector or barley *FCA* whereas in wild type plants, the *FLC* transcripts could not be detected (Fig. 4.22 E).

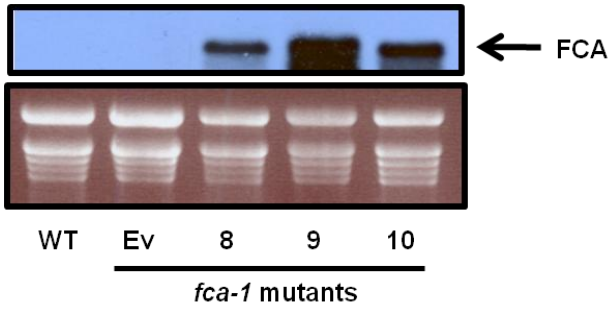
A.


WT
Ev
8
9
10
fca-1 mutant complemented with *HvFCA*

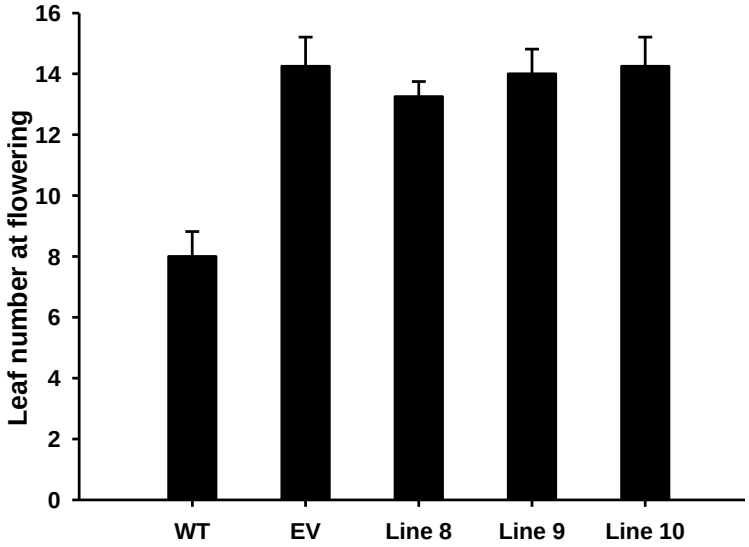
B.


Days to bolting
25
20
15
10
5
0
WT
EV
Line 8
Line 9
Line 10

C.


FCA
WT
Ev
8
9
10
fca-1 mutants

D.



E.

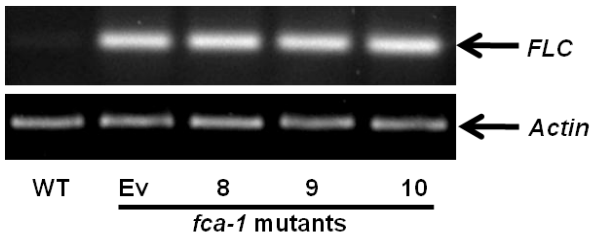


Figure 4.22. Study of recovery of late flowering phenotype of *fca-1* mutant complemented with barley *FCA*. WT-Wild type (Ler); Ev- *fca-1* mutant complemented with empty vector construct; lines 8, 9 and 10 are *fca-1* mutant complemented with *HvFCA* construct. The error bars on figures represent standard deviation from 8-10 plants from two generations. **(A)** Presence or absence of flowering in various lines. **(B)** Days to bolting in various lines. **(C)** Northern blot showing detection of barley *FCA* transcripts in various lines. **(D)** Leaf number at flowering in various lines. **(E)** Gel blot showing detection of FLC transcripts through reverse transcription PCR in various lines.

5.0 DISCUSSION

5.1 *ABAP1* is a fragment of *FCA* and may not exist independently

The proposal that ABAP1 is an ABA-binding protein led to the current investigation to find its physiological function and to trace its origin, as it has high sequence similarity to the flowering time control protein *FCA*. Cloning of barley *FCA* and comparing its amino acid sequence to ABAP1 showed that ABAP1 was identical to the C-terminal half of FCA (Fig. 4.2). Since *FCA* is known to undergo alternative splicing (Macknight *et al.* 1997), the possibility that *ABAP1* might be one of the products formed through splicing was investigated. Northern blot analysis using a probe common to *ABAP1* and *FCA* showed the presence of *FCA-γ* only (Fig. 4.3), even in the presence of ABA, where *ABAP1* should be found in abundance (Razem *et al.* 2004). There has been scepticism about the presence of *ABAP1* as an independent transcript as the closest homologue of *ABAP1* was *FCA* in all studied plant species (Finkelstein 2006). The similarity between the genomic DNA regions upstream of *ABAP1* and the expressed regions of *FCA* (Appendix 2) suggests that *ABAP1* has the same chromosomal origin as *FCA*, ruling out the possibility of *ABAP1* being formed from a different locus on the chromosome. Western blots with anti-idiotypic antibodies recognized ABAP1 recombinant protein but did not react to any protein from the ABA-treated or untreated cytosolic or microsomal fraction of barley aleurone layer protein (Fig. 4.4). However, earlier studies detected similar band intensities with 25 µg of tissue protein and as high as 1 µg recombinant protein suggesting that ABAP1 is

present in quantities as much as 4% of total loaded protein (Razem *et al.* 2004). The evidence from the current work demonstrates that the anti-idiotypic antibody used in the previous studies (Razem *et al.* 2004) and this study can detect as low as 10ng (0.04% of 25 µg) of recombinant ABAP1 in samples with or without HIS tag and still cannot detect any protein from 25 µg protein loaded from tissue fractions (Fig. 4.4) suggesting that contamination of recombinant ABAP1 was present in the tissue protein lanes in the studies performed by Razem *et al.* (2004). The existence of ABAP1 protein was also tested by anti-ABAP1 antibody which detected multiple proteins including a 52 kDa protein from leaf and various seed tissues (Fig. 4.5). Since ABAP1 was shown not to be present in tissues other than seeds (Razem *et al.* 2004), it is most likely that the 52 kDa protein identified by anti-ABAP1 antibody is not ABAP1 as it identifies a protein that is present in all tissues including the leaves.

The current evidence suggests that *ABAP1* is a truncated fragment of *FCA* and may not exist independently. In this study, despite repeated attempts, cloning the 3' region of *ABAP1* gene fragment (which makes it a full length *FCA*) did not yield results, probably due to the presence of regions high in CG repeats which make it very hard to perform 3' RACE. Northern blot analysis that showed presence of *ABAP1* and *FCA* as independent transcripts (Razem *et al.* 2004) can be explained by binding of the *ABAP1* probe to 18S and 28S components of ribosomal RNA which migrate at the same molecular size as *ABAP1* and *FCA* respectively. The Northern blot in earlier work (Razem *et al.* 2004) appears to be over-saturated, suggesting improper washing of the radio-labelled membrane

prior to exposure. Finally, the absence of ABAP1 protein in Western blots further suggests that *ABAP1* is, in fact, an artefact resulting from a nucleotide fragment of *FCA* isolated during the cloning process.

5.2 Conservation and divergence between dicot and monocot *FCA* gene

The RNA-binding protein FCA was cloned and characterized as a protein involved in a photoperiodic response in both dicots and monocots (Koornneef *et al.* 1991, Lee *et al.* 2005, Macknight *et al.* 1997). Alternative splicing of *FCA* is conserved in both rice and Arabidopsis, forming four isoforms (α, β, γ and δ) of which only γ forms a functional protein (Du *et al.* 2006, Lee *et al.* 2005, Macknight *et al.* 1997). The constitutive over-expression of rice *FCA* partially rescued the delayed flowering phenotype of *fca-1* mutant in Arabidopsis, suggesting overlapping roles of the two proteins (Lee *et al.* 2005).

The barley FCA amino acid sequence showed a high degree of homology with rice, wheat, and Arabidopsis within the two RRMs and the WW domain (Fig. 4.6). The FCA protein in barley and other monocots also contain additional domains, namely regions rich in glycine at the N-terminal end, RGG motifs and a poly-glutamine domain at the C-terminal end of the WW domain. These may modify the function of the monocot FCA compared to the dicot FCA. A host of RRM-containing proteins have additional domains like RGG that can modify the functions of RNA binding proteins (Siomi and Dreyfuss 1997). The N-terminal glycine residues that are present in barley FCA but absent in Arabidopsis FCA are known to modify the role of G-Proteins in signalling (Mumby *et al.* 1990)

probably by change in the folding properties (Krieger *et al.* 2005). Since the function of FCA in Arabidopsis is closely tied to the protein it interacts with (Liu *et al.* 2007, Manzano *et al.* 2009, Quesada *et al.* 2003), the variation in protein folding caused by the presence of glycine-rich regions may cause variation in functionality of barley FCA. The poly-glutamine tract downstream of the WW domain consists of two residues of glutamine in Arabidopsis compared to seven in barley and nine in rice (Fig. 4.6). There is evidence to suggest that the length of the poly-glutamine tract directly C-terminal to the WW protein-protein interaction domain affects the FCA-FY interaction, altering the auto-regulation of *FCA* (Lindqvist *et al.* 2007). Constitutive expression of rice FCA (with nine glutamines C-terminal to the WW domain) in Arabidopsis, however, did not regulate the endogenous levels of *FCA-γ*, contrary to what was observed when Arabidopsis *FCA* (with two glutamine residues) was constitutively expressed in Arabidopsis (Lee *et al.* 2005, Macknight *et al.* 1997). Given the fact that the *FCA*-FY interaction is now being considered to be transient (Manzano *et al.* 2009), the differences in protein motifs between monocot and dicot FCA may have led to variation in how FCA interacts with its protein partners resulting in altered function.

5.3 Regulation of *FCA* in barley embryos

5.3.1 *FCA* transcript levels during embryo maturation and germination

The work on *ABAP1* showed that it was up-regulated with ABA treatment in barley seed tissue (Razem *et al.* 2004). Since the existence of ABAP1 is now

questionable, the current investigation focussed on the effect of ABA on the full length *FCA* transcripts and its regulation during embryo maturation. Studies on Arabidopsis FCA suggest that female gametophytic development and early embryonic development are susceptible to malfunctions in FCA (Baurle *et al.* 2007). A recent report shows that *FCA* transcripts are up-regulated during bud dormancy in poplar (Ruttink *et al.* 2007). The *FCA* transcripts could be detected from a very early stage of embryo development (2 DAP) up to the mature desiccated embryo (Fig. 4.8 A & B). Although the *FCA* transcripts did not show major changes in amount, band intensity analysis showed that the *FCA* transcripts are slightly higher in desiccated embryos compared to earlier stages (Fig. 4.8 B).

There are common signals that regulate the transition from one developmental stage to another in plants (Baurle *et al.* 2007). Arabidopsis FCA plays a major role in the transition from the vegetative to reproductive phase (Macknight *et al.* 1997). During germination, barley *FCA* transcripts declined by 30% (Fig. 4.9 B). In the presence of ABA, the decline in *FCA* transcripts during germination was lower during the first 16 hrs. (25%) compared to untreated embryos, after which the transcripts remained stable suggesting that ABA inhibits the down-regulation of *FCA*.

The constant presence of barley *FCA* during embryo development and maturation suggests that FCA is required during these stages. It is surprising that although ABA increases during desiccation of the embryo (Suzuki *et al.* 2000) leading to increased *FCA* transcripts, this study could detect only slight changes

in *FCA* transcripts during late maturation stage of the embryos (Fig. 4.8 B) suggesting that *FCA* may have tighter control on its transcript regulation during embryo development. Since the *FCA* regulation in embryos has never been studied before, a detailed analysis of *FCA* regulation during embryo development using more sensitive methods (real-time PCR) may shed more light on its regulation.

5.3.2 ABA and salt stress up-regulate barley *FCA*

Barley *FCA* transcripts were induced by ABA and salt stress in germinated barley (monocot) embryos and seedlings (Fig. 4.11 A-C). ABA increased *FCA* transcripts but this increase was not dose dependent on the range of ABA concentrations used (Fig. 4.11 A). The response of *FCA* to ABA may be saturated by 2 µM and hence *FCA* transcript analysis at range below 2 µM ABA is needed to confirm this hypothesis. Alternatively, ABA may affect *FCA* through an indirect pathway. The regulation of Arabidopsis *FCA* occurs at the post-transcriptional level where its transcripts are auto-regulated by its own transcripts (Quesada *et al.* 2003).

Repeated efforts to clone the *FCA* promoter from barley were unsuccessful, but the rice *FCA* promoter has four ABA response elements (ABRE) compared to one in Arabidopsis (Appendix 4 & 5). None of the two promoters contained coupling elements (CE) which are known to be required for functioning of a single ABRE (Shen and Ho 1995). The coupling elements and ABRE are functionally equivalent for ABA responsiveness (Hobo *et al.* 1999). Two

or more ABRE elements are sufficient for ABA response as ABRE can also function as coupling elements when present in multiple copies (Gomez-Porras *et al.* 2007). The two promoter sequences contain elements for dehydration responsiveness, light responsiveness, pathogen responsiveness and GA responsiveness (Appendix 4 & 5). Based on the experimental results regarding *FCA* up-regulation by ABA and stress and the *in silico* analysis of *FCA* promoters from rice, it can be suggested that the barley *FCA* gene is regulated by ABA. The fact that the Arabidopsis *FCA* promoter is non-responsive to ABA could be due to the presence of a single ABRE without any coupling element compared to the multiple ABRE in monocot *FCA* promoter.

ABA controls the RNA-dependent RNA polymerase required for the synthesis of siRNA (Yang *et al.* 2008) and regulates stress responses through DNA methylation and siRNA interference (Chinnusamy *et al.* 2008). *FCA* requires siRNA for locus-specific chromosomal modification (Baurle *et al.* 2007). Phase transition, be it vegetative to reproductive or embryonic to post-embryonic, requires major changes in gene regulation. Gene expression during seed development and germination are regulated by ABA through networks of transcription factors belonging to the B3 domain (ABI3/VP1; LEC2 and FUS3), APETELA2 (ABI4), bZIP (ABI5) and HAP3 subunit of CCAAT binding factor (LEC1) (Finkelstein *et al.* 2002). In addition, *ABI3* and *ABI5,* negative regulators of germination in dicots and monocots, undergo post-transcriptional modification through alternative splicing (McKibbin *et al.* 2002; Zou *et al.* 2007).

5.4 Barley *FCA* affects ABA-responsive promoters

Barley aleurone layer protoplasts transiently expressing *Em* and *VP1* gene promoters showed that both promoters are activated by transiently expressing barley *FCA* and this activation was further enhanced by addition of ABA (Fig. 4.12 & 13). Similar results were obtained when the experiments were carried out with truncated *FCA* (lacking RRM) (Fig. 4.14 & 15). The *Em* promoter was responsive to ABA but the *VP1* promoter showed minimal response to applied ABA (Fig. 4.13). The *VP1* promoter construct (provided by Dr. P. Becraft) is non-responsive to ABA (Cao *et al.* 2007) but Dr. Becraft's lab obtainined a 1.5 – 2.0 fold increase with its application in maize embryos (personal communication). The effect of anti-sensing *FCA* had no significant effect on the two gene promoters in the absence of ABA but down-regulation of the promoters occurred in the presence of ABA. The difference in the response is likely due to the levels of GUS activity being too low to show significant further down-regulation (Fig. 4.12-15).

Transient expression of *FCA* increased *VP1* transcripts levels in the absence of ABA (Fig. 4.16); however, no significant difference was found in the levels of *VP1* transcripts in the presence of ABA compared to control conditions (Fig. 4.17). The gene *VP1* is known to be up-regulated by ABA (Cao *et al.* 2007) and it is likely that the effect of ABA is more pronounced and homogeneous than the response resulting in a masking of the effect caused by bombardment. This result should not be confused with results from promoter studies. The promoter response (represented by GUS activity) comes only from the transformed cells and is normalised with a transformation control (LUC). However, in transient

expression studies using embryos, the transcripts represent contributions from both transformed and non-transformed cells. Hence, the effect of ABA is more pronounced and homogenous negating the effect of transient expression. *SLN1*, the only gene that encodes a DELLA protein in barley is regulated at the transcriptional level by light signalling and post-transcriptionally by ABA (Gubler *et al.* 2002, Oh *et al.* 2007). DELLA proteins affect flowering in an ABA-dependent manner (Achard *et al.* 2006). The effect of *FCA* on the levels of *SLN1* in the presence of ABA (Fig. 4.17) raises the interesting possibility that *FCA* is the common factor influencing DELLA protein expression in both flowering and germination which, however, needs to be tested.

The observation that expression of *FCA* can affect the *Em* gene promoter suggests that either the product of *FCA* can act directly on this promoter or affected signalling intermediates that in turn act on the *Em* gene promoter. The expression of *FCA* also affected *VP1* transcripts known to induce Em gene expression (Hattori *et al.* 1995). The RRM region of barley *FCA* is not required for the observed effects on the *Em* gene promoter suggesting that the effects are occurring due to interaction of FCA with components not requiring the RRM region for interaction. For example, FCA is known to interact with the SWI/SNF class of chromatin remodelling complexes through a domain present at the C-terminal of FCA and not involving the WW domain (Sarnowski *et al.* 2002). FCA, through its WW domain, also interacts with FY, a 3' poly-adenylation factor that, amongst other effects, causes alternative splicing of *FCA* (Quesada *et al.* 2003) and probably other targets (Henderson *et al.* 2005). It is possible that *FCA* affects

the alternative splicing of *VP1* causing the changes in transcript levels of *VP1* which in turn affects the *Em* gene promoter.

5.5 *FCA* may cause organ level changes due to generation of transmissible signals

The most interesting finding from the transient expression studies was that the transformation of *FCA* had effects far from the site of transformation. *FCA* could affect *VP1* transcript levels not only at the site of transformation but also away from it (Fig. 4.19 A & B). FCA acts through small RNAs (Baurle *et al.* 2007), that are highly labile in plant system during development (Chitwood *et al.* 2009), and may be responsible for transmission of *FCA* signals. Another explanation to the transmission of signal by FCA can be via FT. FCA affects FT through FLC (Macknight *et al.* 1997). FLC affects seed germination in Arabidopsis (Chiang *et al.* 2009) and FT, the mobile element known as florigen (Corbesier *et al.* 2007), affects bud dormancy (Bohlenius *et al.* 2006).

5.6 Localization of barley FCA is due to a nuclear localization signal

The nuclear localization of FCA makes it a possible candidate to fine tune gene regulation via chromatin modifications (Quesada *et al.* 2003). Barley FCA also localized to the nucleus in onion epidermal cells (Fig. 4.20 A & B). The results presented in the current study also confirm that the nuclear localization of barley FCA is due to the presence of a Nuclear Localization Signal (NLS) present after the RRM in barley FCA but is absent from Arabidopsis FCA (Fig. 4.22 A-H).

The FCA fragment lacking the RRM but retaining the NLS also localized to the nucleus suggesting that the nuclear localization of barley FCA is due to the presence of NLS and not due to RRM. The presence of truncated FCA in places other than the nucleus may be due to the fact that the truncated FCA still contains the WW domain and may interact with other proteins while it is being transported to the nucleus. It is also possible that while most of the truncated FCA goes to the nucleus, it is still a truncated protein and a part of it may get targeted to the vacuoles for degradation. The study of localization of FCA in barley aleurone layer protoplast should also be carried out to understand if the localization of truncated FCA in non-nuclear compartments is due to its WW domain or due to overloading of the FCA/trunk FCA in aleurone layer cells.

5.7 *FCA*-mediated control of flowering between barley and Arabidodpsis is not conserved

The complementation study to rescue the late flowering phenotype of the *fca-1* mutant showed no effect of barley *FCA* on flowering in Arabidopsis (Fig. 4.22 A-D). Furthermore, the constitutive expression of barley *FCA* did not result in the down-regulation of *FLC* (Fig. 4.22 E). This is in agreement with the study of Lee *et al.* (2005) in terms of the monocot *FCA* effect on *FLC* but differs on the partial rescue of flowering phenotype by rice (monocot) FCA. As discussed in section 5.2, this is additional evidence suggesting that the presence of additional domains on barley FCA likely modifies its function. The results also support the

suggestion that monocot FCA has divergent functions compared to dicot *FCA* (Lee *et al.* 2005).

6.0 GENERAL DISCUSSION

This thesis research was initiated to study the physiological function of an ABA binding protein, ABAP1, isolated from barley aleurone layer tissue and shown to be induced by ABA (Razem *et al.* 2004). *ABAP1* showed very high sequence similarity to rice *FCA*, a gene well known to be involved in flowering in Arabidopsis (Macknight *et al.* 1997). There was scepticism about the independent existence of ABAP1 (Finkelstein 2006). The ABAP1 protein was known only in barley and its homologue in all other studied plant species was an RNA binding protein, FCA. Cloning of *FCA* from barley established that *ABAP1* and barley *FCA* had regions in which they had identical sequences. In protein sequence comparision, ABAP1 consisted of the C-terminal half of FCA. Analysis of *FCA* and *ABAP1* transcripts and proteins suggested that ABAP1 did not exist and that *FCA* was the only detectable transcript in barley aleurone. This resulted in a refocusing of the thesis work to characterize barley *FCA* and to examine its physiological function in both barley and Arabidopsis. This study has led to a number of contributions to the existing knowledge of *FCA* in particular.

The various lines of evidence from this study suggest that barley *FCA* not only affects the transition to flowering but has a broader influence in affecting other reproductive processes such as germination. The steady presence of *FCA* during embryo development and its down-regulation during transition from non-germinated to germinated state suggest that it is required during reproductive development. This broader role of *FCA* in reproductive development is not

uncommon for autonomous pathway genes. The floral transition inhibitor FLC, which is also a target of FCA, was recently shown to regulate germination in Arabidopsis (Chiang *et al.* 2009). The flowering time regulator, FT which is an indirect target of FCA and FLC, is also involved in regulation of bud dormancy in tree species (Bohlenius *et al.* 2006). At the cellular level, genes containing RRM or WW domains are known to affect a number of pathways (Baurle *et al.* 2007). Sequence differences in FCA between barley and Arabidopsis result in barley FCA not complementing Arabidopsis FCA suggesting that the function of FCA has evolved differently in monocot and dicot systems. This is also evident from work with rice FCA where it did not affect *FLC* levels in Arabidopsis (Lee *et al.* 2005).

Barley *FCA* affects genes that are alternatively spliced as shown by the observed changes in the levels of transcription factors and other genes that are involved in ABA responses. FCA interacts with FY, a 3' polyadenylation factor, to regulate its own transcript levels (Quesada *et al.* 2003). This interaction, once thought to be stable, is now known to be transient, affecting the organization of splicing machinery on the FY protein (Manzano *et al.* 2009). It is hypothesised that FCA senses the changes in cell surroundings which in turn affects its interaction with FY, altering the organization of splicing machinery on FY (Manzano *et al.* 2009). Recent studies in our lab have shown that FY is involved in regulation of germination in Arabidopsis (Jiang *et al.,* unpublished result) suggesting that FCA can affect the alternative splicing of various targets depending upon tissue or cell environment.

The RRM domain of FCA does not seem to be required for its effect on ABA responsive promoters. Although possible, it is unlikely that FCA binds to the promoters of the ABA responsive elements. FCA is known to interact with an SWI/SNF class of chromatin remodelling factors through its C-terminal region other than WW domain (Sarnowski *et al.* 2002). One such protein is PKL (Ogas *et al.* 1999) which is known to affect the promoters of *VP1/ABI3* and *ABI5* (Perruc *et al.* 2007). This again hints that the main targets of FCA are the transcription factors that mediate ABA signalling, be it through chromatin modification and/or alternative splicing.

Another interesting outcome of the study was the widespread effect of *FCA* far from the site of transformation. Although preliminary, it does support the earlier findings that FCA can affect its targets though generation of labile signals (Furner *et al.* 1996, Manzano *et al.* 2009). FCA works with small RNAs that are highly labile in plant systems to cause chromatin modification (Baurle *et al.* 2007). In the flowering pathway, FCA indirectly affects the flowering time regulator gene (FT) that is known to be labile as a protein (Corbesier *et al.* 2007) and is known to induce bud dormancy in trees (Bohlenius *et al.* 2006).

From the current thesis work, it can be hypothesised that various environmental stresses cause increase in ABA levels in plants (Christmann *et al.* 2005) which in turn affects FCA levels (Fig. 6.1). FCA acts on the transcription factors involved in ABA signalling and dormancy either through pre-transcriptional events (chromatin modification) or post-transcriptional events (alternative splicing) causing pre-mature flowering or inhibition of germination (Fig. 6.1). This work puts

in place the building blocks for understanding the commonality between floral transition and germination and the epigenetic regulation of these events.

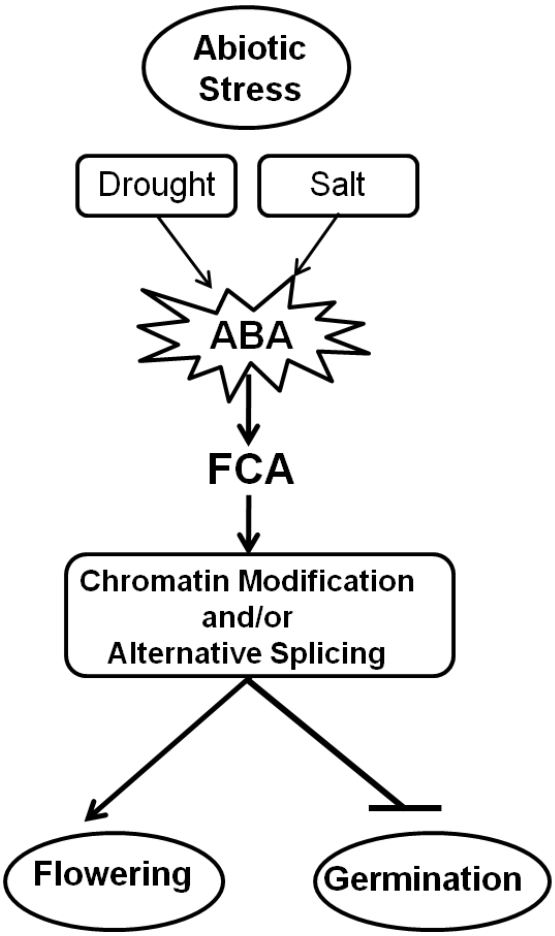


Figure 6.1. A hypothetical model of the mode of action of barley FCA in regulating flowering and dormancy.

7.0 SUMMARY OF CONCLUSIONS

The current study aimed to understand the origin and functional significance of the ABA binding protein ABAP1 resulting in the following conclusions:

1. ABAP1 is identical to the C-terminal half of barley FCA and is most likely a truncated FCA which was not completely cloned as a full length transcript in earlier experiments.

2. The comparison of monocot and dicot FCA shows that monocot FCAs contain additional domains that can modify its function. The functional analysis of barley *FCA* into Arabidopsis suggests that this gene is diversified into the two systems.

3. Barley FCA affects ABA regulated genes that are involved in regulation of dormancy and germination in barley. A localized alteration in levels of *FCA* is able to induce organ level change in FCA modulated events.

8.0 REFERENCES

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9.0 APPENDICES

9.1 Appendix 1

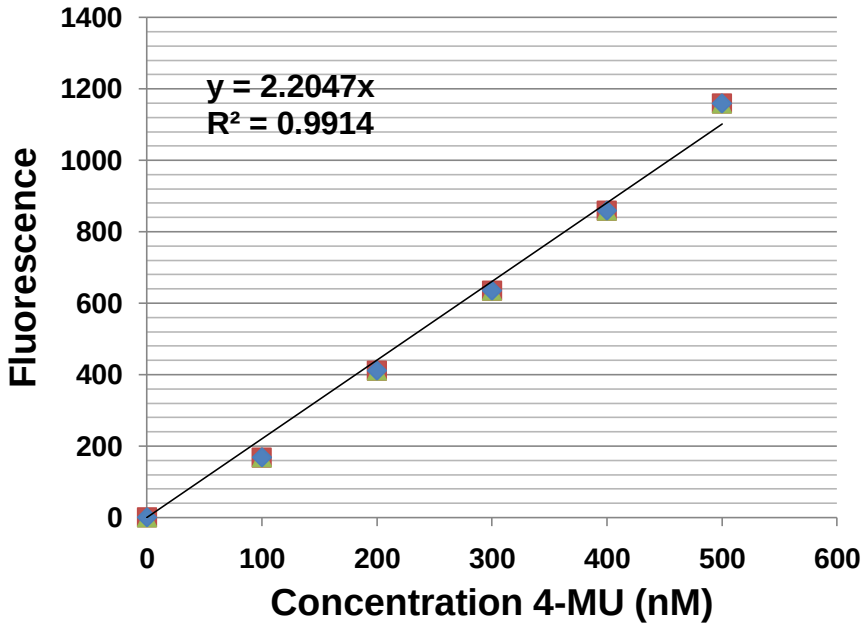

Figure 9.1. Standard curve for 4-MU activity.

9.2 Appendix 2

gb|FJ188402.2| Hordeum vulgare flowering time control protein (*FCA*) mRNA, complete cds Length=2647

GENE ID: 100286757 *FCA* | flowering time control protein [Hordeum vulgare]

Sort alignments for this subject sequence by:

E value Score Percent identity

Query start position Subject start position

```
 Score =  159 bits (86),  Expect = 4e-35
 Identities = 86/86 (100%), Gaps = 0/86 (0%)
 Strand=Plus/Plus
Query  960   AGGCTGTTGTTTTGTTAAATATGCTACTTCCGAAGAGGCCGAGAGAGCCATCAGAGCTCT  1019
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  568   AGGCTGTTGTTTTGTTAAATATGCTACTTCCGAAGAGGCCGAGAGAGCCATCAGAGCTCT  627

Query  1020  GCATAACCAGTGCACTATACCCGGGG  1045
             |||||||||||||||||||||||||
Sbjct  628   GCATAACCAGTGCACTATACCCGGGG  653
 Score =  130 bits (70),  Expect = 3e-26
 Identities = 70/70 (100%), Gaps = 0/70 (0%)
 Strand=Plus/Plus
Query  1751  AGATTTTTGCTCCTTTTGGTCATGTGGAAGATGTTTACATCATGAAAGATGGCATGAGGC  1810
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Sbjct  771   AGATTTTTGCTCCTTTTGGTCATGTGGAAGATGTTTACATCATGAAAGATGGCATGAGGC  830

Query  1811  AGAGCCGAGG  1820
             ||||||||||
Sbjct  831   AGAGCCGAGG  840
 Score =  128 bits (69),  Expect = 1e-25
 Identities = 69/69 (100%), Gaps = 0/69 (0%)
 Strand=Plus/Plus
Query  1599  GGGTCTATTGAGCACAAATTGTTTGTTGCATCACTGAATAAGCAGGCAACTGCAAAGGAG  1658
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  704   GGGTCTATTGAGCACAAATTGTTTGTTGCATCACTGAATAAGCAGGCAACTGCAAAGGAG  763

Query  1659  ATAGAAGAG  1667
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Sbjct  764   ATAGAAGAG  772
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 Identities = 54/54 (100%), Gaps = 0/54 (0%)
 Strand=Plus/Plus
Query  1124  GGCGATGGGCCCTGTTCAGGTTAGATATGCTGATGGTGAAAAGGAGCGTCATGG  1177
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Sbjct  652   GGCGATGGGCCCTGTTCAGGTTAGATATGCTGATGGTGAAAAGGAGCGTCATGG  705
 Score = 97.1 bits (52),  Expect = 3e-16
 Identities = 52/52 (100%), Gaps = 0/52 (0%)
 Strand=Plus/Plus
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Sbjct  838   AGGCTGTGGCTTTGTCAAATTCTCATCAAAAGAACCTGCACTTGCAGCCATG  889
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Figure 9.2. Sequence similarity search (NCBI Blast) for genomic DNA clone upstream of *ABAP1* showing sequence similarity with expressed regions of barley *FCA*.

9.3 Appendix 3

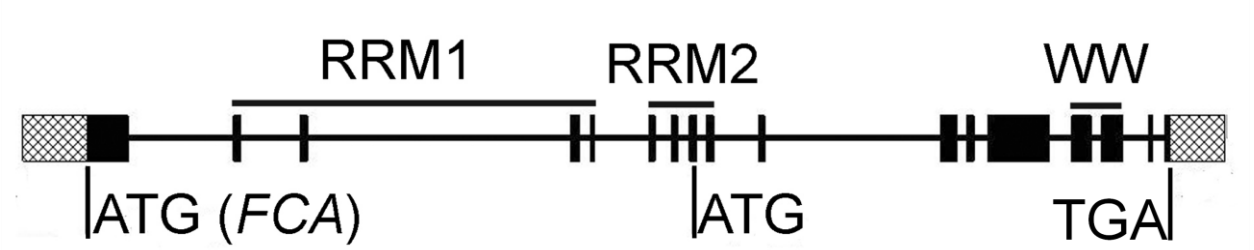

Figure 9.3. Diagrammatic representation of exons used to generate a truncated *FCA* fragment. The second ATG at exon 8 was used as the initiation codon and TGA as the stop codon.

9.4 Appendix 4

gtcatggttatatccgagctagttgtgtcctcatcatcttgcatgagatttaaaatactctagag
tatgatgatgatatgccatagtggatgatgatgtgacacagttacatgttgagctttaggagtta
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gggacggccgattcggccgcggcccttctcgctggtcgtcc

***ABRE (4 hits)**	**Etiolation-induced expression/ABA/dehydration response element**
AACACOREOSGLUB1	Endosperm specific gene expression
ARR1AT	Cytokinine responsive elements
ATHB1ATCONSENSUS/CORE	Leucine Zipper binding elements
CCAATBOX1	**Heat Shock Element**
EBOXBNNAPA	Required for high promoter activity of seed storage protein genes
GATABOX	**Light regulation and tissue specificity**
GCCCORE	Jasmonate signaling and pathogen response
GT1CONSENSUS	Salisylic acid and light regulation
***MYB1AT/MYCCONSENSUSAT**	**Dehydration and stress response**
WBBOXPCWRKY1	WRKY protein binding site
WBOXNTERF3	**Wounding response**
WRKY71OS (5 hits)	**WRKY71 binding element for repression of GA signalling**

Figure 9.4. Rice *FCA* promoter sequence shows the presence of various motifs as analysed by the promoter analysis tool PLACE. The underlined sequences represent ABA response elements.

9.5 Appendix 5

gatctaggtgaaattaatctgaagtttagaaatagattttcttggaacttcggagaaaatatgct
tcactcaacttttttttggtgctatatgaacaaagataatggtcatatgaatgtaaacgtgtttt
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gaattggaattgggggcggaggaggaggtaatggtggtcgacgctgggaagatgacactcctaag
gattttgatggtcccggagatggaggtttccggcag

***ABRE (1 hit)**	**Etiolation-induced expression/ABA/dehydration response element**
CIACADIANLELHC	Circadian and light response elements
CRTDREHVCBF2	CBF2 binding element (Temperature)
GATABOX	**Light regulated expression**
GCCCORE	Pathogen responsive genes
HEXMOTIFTAH3H4	**Histone binding**
MYB1AT	Dehydration response elements
MYBCORE	Dehydration and water stress; Flavonoid synthesis
PYRIMIDINEBOXOSRAMY1A	GA response elements
WRKY71OS (5 hits)	**WRKY71 binding element for repression of GA signalling**

Figure 9.5. Arabidopsis *FCA* promoter sequence shows the presence of various motifs as analysed by the promoter analysis tool PLACE. The underlined sequences represent ABA response elements.

9.6 Appendix 6

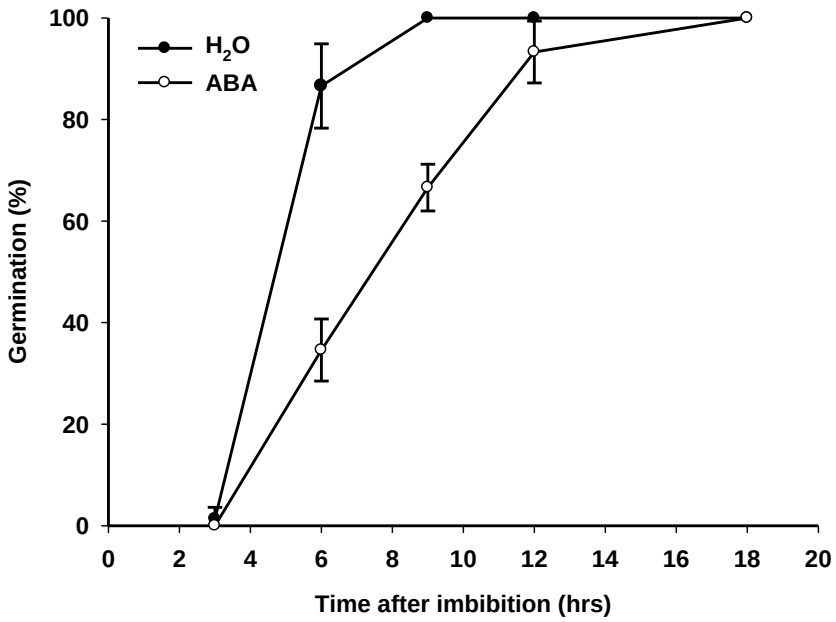

Figure 9.6. Germination of barley cultivar McLeod excised embryos (2007 harvest) in presence of water or 10 µM ABA. The error bars represent standard deviation from three replicates.