TOWARD FUNCTIONAL CHARACTERIZATION OF *Triticum aestivum* *WFCA*-CODING SEQUENCES

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ABSTRACT

Flowering is a critical step in the plant life cycle. If flowering occurs too early or too late, seed production suffers. Flowering is regulated through numerous flowering repressors. As long as these repressors persist, the plant will remain in a vegetative growth stage. Some plants possess two separate genetic pathways, the autonomous pathway and the vernalization pathway, that promote the transition to flowering through stable downregulation of flowering repressors. Once the plant achieves floral competence, it will flower under inductive environmental conditions.

In *Arabidopsis*, *FCA* is a key autonomous pathway gene, acting with *FY* to promote the floral transition. Recently, gene sequences resembling *FCA* were cloned from hexaploid wheat (*Triticum aestivum*) and designated as *WFCA*. WFCA shows numerous similarities to the FCA peptide, especially regarding three key regions: two RNA Recognition Motifs and the WW domain. This study seeks to determine if *WFCA* genes function similar to *FCA* by determining if they are able to complement the *fca-1* mutant of *Arabidopsis thaliana*.

T1 progeny from an *Arabidopsis fca-1* plant transformed with *WFCA* were grown without vernalization and assayed for the final leaf number (FLN). The late flowering *fca-1* control plants bolted with an average FLN of 14.8 while the T1 population had an average FLN of 14.3. Although the numerical difference is slight, the results are statistically significant, and suggest that *WFCA* genes may have some degree

of flowering promotion activity in *Arabidopsis*. The lack of strong complementation may be due to divergence of the *WFCA* genes from their *Arabidopsis* counterparts. With increasing evidence for divergence in flowering promotion between monocot and dicot species, the development of a robust monocot model system appears to be critical to provide a good framework to assist studies of the particular nuances of the monocot flowering process.

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LIST OF ABBREVIATIONS

aa, amino acid

ABA, abscisic acid

APS, ammonium persulfate

bp, base pair or base pairs

BSA, bovine serum albumin

cDNA, complementary DNA

CTAB, Hexadecyltrimethylammonium bromide

CTP, putative C-terminal peptide of *FCA*-like genes

DEPC, di-ethyl Pyrocarbonate

EDTA, ethylenediamine tetra-acetic acid

EMF, *EMBRYONIC FLOWER* genes

FLC, *FLOWERING LOCUS C*

FRI, *FRIGIDA*

GA, gibberellic acid

gDNA, genomic DNA

GUN, *Escherichia coli* encoded β- glucuronidase fused to nptII

GUS, glucuronidase

IPTG, Isopropyl β-D- thiolgalactoside

kb, kilobasepairs

kDa, kilodaltons

LB, Luria-Bertani media

LD, Long Day photoperiod (16h light, 8h dark)

Mb, megabases

MCS, Multiple Cloning Site

nptII, neomycin phosphotransferase II

nt, nucleotide(s)

PAGE, Poly-Acrylamide Gel Electrophoresis

PCR, Polymerase Chain Reaction

RRM, RNA Recognition Motif

SD, Short Day photoperiod (8h light, 16h dark)

SDS-PAGE, Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis

SSC, Sodium Chloride and Sodium Citrate Solution

TR, tri-nucleotide repeat

TEMED, (N,N,N,N-Tetramethyl-Ethylenediamine)

wt, wild-type

WW, WW Domain, a protein-protein interaction domain

X-Gluc, 5-bromo-4-chloro-3-indolyl-β-D-glucuronide

INTRODUCTION

This project concerns the functional characterization of genes putatively affecting flowering in hexaploid wheat. Earlier, two different full-length genome-specific *WFCA* genes (called *WFCA*, for Wheat orthologs of *FCA*) had been cloned, along with two partial cosmid clones and numerous cDNAs produced from the amplification of both mRNA and genomic DNA (M. Gao, T. Hoffman, A.E. Limin, D.B. Fowler, and G. Selvaraj, unpublished results). The *WFCA* genes were mapped to the long arms of the group 5 chromosomes, and genome-specific sequence variations were detected and characterized. Based on numerous observed variations in *WFCA* DNA sequences and the mapping of the *WFCA* genes to the group 5 chromosomes, it had been postulated that the variation in flowering behaviour between spring and winter wheats might be related in part to the variation in the level of functional *WFCA* genes (M. Gao, personal communication). The group 5 chromosomes of wheat have been shown to strongly affect flowering time, vernalization response, low temperature tolerance, frost tolerance, growth habit, grain hardness, ear morphology, and plant height (Kato et al., 1999; Snape et al., 2001; Limin and Fowler, 2002).

Studies involving heterologous complementation can provide a strong experimental evidence for conservation of gene function, and are particularly illustrative if the putative genes are introduced into a well-studied and characterized plant system. *Arabidopsis thaliana* is the most commonly used model system in plant research today,

and has been extensively studied in terms of its flowering behaviour. The flowering behaviour and biological function of the *FCA* gene was first characterized in *Arabidopsis* (Koornneef et al., 1991; Macknight et al., 1997). Although the role of *FCA* in flowering promotion is still not fully understood, it has been demonstrated to be a vital part of the autonomous flowering pathway in *Arabidopsis* (Macknight et al., 1997). If *FCA* function is disrupted, mutant plants show a delay in flowering in the absence of vernalization treatment. This flowering delay results in the continuation of vegetative growth, producing more rosette leaves prior to bolting in comparison to the wild-type Arabidopsis plant (Koornneef et al., 1991). This cumulative number is referred to as the Final Leaf Number (FLN). The *Arabidopsis* system, due to the extensive number of studies characterizing its flowering behaviour, and several studies targeting *FCA* in particular, is an ideal system to allow us to determine if the *WFCA* genes are functional orthologs of the *FCA* genes, or if they may simply resemble *FCA* while serving a different purpose in the wheat system (Jack, 2004; Komeda, 2004; Parcy, 2005).

It is often difficult to directly characterize the genetic interactions in *Triticum aestivum* due to two major complicating factors: its large genome size, and its polyploid genome. A single wheat genome is estimated to consist of 16 000 megabases (Mb) of DNA contained in 21 chromosomes, and the combination of three functional genomes in *Triticum aestivum* results in a high level of repetitive DNA and potential epistatic interactions, providing substantial difficulties in locating genes as loss-of-function mutations in all three homeologous genes would be rare, and therefore a phenotype would not be seen as readily as in a simpler organism (Hay and Ellis, 1998; Sandhu and Gill; 2002; Li et al., 2004). Furthermore, the group 5 chromosomes of wheat complicate matters as comparative genomic studies have shown that the group 5 chromosomes have

synteny with various regions of rice chromosomes 3, 9, 11, and 12, as well as several regions of undetermined or broken synteny (Sarma et al., 2000). This complex organization of the wheat chromosomes is generally attributed to numerous recombination and translocation events during the speciation and development of polyploid wheats (Isidore et al., 2005).

Despite this high level of complexity, there are typically six to eight gene-rich regions on each wheat chromosome, and many key regions of the group 5 chromosomes have been linked with specific regions of the rice chromosomes (Sandhu and Gill, 2002; Kato et al., 1999; Dubcovsky et al., 1998; Sarma et al., 1998). The long arm of the wheat group 5 chromosomes shares synteny with regions of rice chromosomes 3 and 9 (Sarma et al., 2000; Dubcovsky et al., 1998; Danyluk et al., 2003). The *Vrn-A1* locus, which is instrumental in the determination of the vernalization requirement and is modified by the action of the *Vrn-A2* gene, shows strong synteny with rice chromosome 3 (Loukoianov et al., 2005; Yan et al., 2003; Sarma et al., 1998, 2000). It should be noted that the rice plant does not require vernalization treatment to accelerate flowering.

Since no experimental work has been performed to determine if the *WFCA* genes perform similarly to the *FCA* genes in *Arabidopsis thaliana*, the aim of this M.Sc. project was to determine if the *WFCA* genes encode an FCA-like protein by investigating if WFCA is able to complement the *fca-1* mutation in *Arabidopsis*.

LITERATURE REVIEW

The Wheat Plant

Triticum aestivum, also known as bread wheat or common wheat, is a staple monocotyledonous food crop that has been grown worldwide for centuries due to its extremely broad adaptation to a wide range of environments (Heyne, 1987). The main food product from wheat is the grain kernel borne on aerial culms bearing terminal spikes. Many of the wheat varieties grown today are semidwarf varieties that produce shorter culms and higher grain yields (Peng et al., 1999).

In Canada, wheat is the most common crop produced. In the last 5 years, over 10 million hectares of wheat have been seeded annually (Statistics Canada website, www.statcan.ca). Total wheat exports for 2004 amounted to revenue in excess of $3.5 billion dollars, a total of 11.4% of all Canadian agricultural export revenue (Statistics Canada website, www.statcan.ca). In terms of the total Canadian export revenue for 2004, wheat exports account for 0.8% of the total ($429 billion dollars) (Statistics Canada website, www.statcan.ca).

Wheat has become a vital food source for several reasons. The wheat kernel is high in carbohydrates and fibre, and is also a source of protein. Wheat flour is also easily digested. Wheat flour is especially notable for the special properties of its gluten, which is instrumental in the production of leavened bread. The insoluble gluten in wheat flour forms numerous cells that retain the carbon dioxide bubbles produced by yeast during

fermentation, causing the dough to rise and providing a lighter, fluffier texture (Heyne, 1987). Originally consumed mainly as a porridge or in unleavened bread, wheat and wheat flour is now used in a wide variety of food products, including bread loaves, flatbreads, cakes, buns, pasta, and crackers. In addition, there are several value-added uses for wheat and wheat fibre, including animal feed, the production of specialized food products such as wheat germ, and in industrial applications including the production of paper and ethanol.

In Canada, wheat grain is typically divided into seven different market classes, depending on its growth habit (spring or winter), hard or soft kernel, the kernel colour (red or white), and the type of wheat (common or durum). Kernel hardness is a reflection of its protein to starch content, with harder wheats containing more protein and soft wheats containing higher levels of starch (Heyne, 1987). When ground, soft wheats produce significantly smaller flour particles than hard wheats (Heyne, 1987). Winter wheats typically have lower protein concentrations than spring wheats, although recent breeding efforts have raised the protein concentration in winter wheats (Heyne, 1987).

Triticum aestivum is a hexaploid species, containing three distinct genomes from three ancestral species. Although the precise origin is not known, hexaploid wheat is thought to have originated in the Mediterranean region around the Fertile Crescent around 6000 B.C.E. (Huang et al., 2002). The ancestry of modern hexaploid wheat is still under debate, but current studies suggest that it likely arose from a hybridization of *Triticum urartu* (AA genome) with a B-genome donor to produce a tetraploid wheat (*Triticum turgidum*; AABB) (Dvorak, 1998; Huang et al., 2002). *T. turgidum* (AABB) later hybridized with *Aegilops tauschii* (DD genome) to produce the ancestral hexaploid *Triticum aestivum* (Huang et al., 2002; Matsuoka and Nasuda, 2004). The B-genome

donor is unconfirmed, but hybridization studies and sequence comparisons have suggested that *Triticum searsii* or a related species was likely the original donor of the B-genome (Nath et al., 1983; Thompson and Nath, 1986). Other studies suggest that there may have been several progenitors of the modern tetraploid and hexaploid wheats (Gu et al., 2004; Caldwell et al., 2004). Based on synteny analysis, all three wheat genomes show a conservation of organization despite their speciation, although there appears to be characteristic differences in transposon frequencies based on variations in repetitive sequences (Snape et al., 2001; Zhang et al., 2004). Today, most commercially grown wheat varieties are hexaploid or tetraploid varieties, as they tend to have a broader climate adaptation and produce larger leaves and kernels (Heyne, 1987).

Vernalization and the Winter Growth Habit

The term "vernalization" is used to refer to the promotion of flowering after a prolonged period of exposure to low temperatures (Chouard, 1960; Danyluk et al., 2003; Finnegan et al., 1998). The major targets of vernalization are the meristematic tissues as only tissues containing actively dividing cells are able to sense and respond to the vernalization treatment (Sung and Amasino, 2004). Vernalization is naturally accomplished through the onset of winter, where the low temperatures and low-light conditions vernalize plants for several months. For this reason, vernalization treatment is often performed under conditions of reduced photoperiod length to mimic the low light levels during winter. Depending on the plant species and its genetics, the need for vernalization treatment can be obligatory, facultative, or non-existent (Finnegan et al., 1998). Flowering promotion due to vernalization is proportional to the length of treatment, until a species-specific genetic requirement has been reached (vernalization

saturation), after which further vernalization treatment will have no effect accelerating the flowering transition (Chouard, 1960; Limin and Fowler, 2002; Prasil et al., 2004). The response to a vernalization treatment varies with species as well, with different lengths and/or minimum temperatures required to produce an optimum vernalization response (Lin et al., 2005).

Winter-annual plants, including the economically important hexaploid wheat *Triticum aestivum*, require a vernalization treatment in order to promote their flowering transition (Prasil et al., 2004). Spring-annual plants do not possess this vernalization requirement, and even if vernalized will flower under promotive environmental conditions at approximately the same time as unvernalized plants (Limin and Fowler, 2002; Prasil et al., 2004). In the absence of vernalization treatment, the time for a winter annual to attain flowering competence is significantly delayed, resulting in an extended period of vegetative growth.

Flowering Promotion in *Arabidopsis thaliana*

The transition from vegetative growth to reproductive growth is a key stage in the life cycle of plants. Due to the sessile nature of plants, it is essential for their survival to balance its physiological development against the need to reproduce. Extended periods of vegetative development allows the plant to accumulate larger stores of vital nutrients and develop increased photosynthetic capacity, develop structural support to allow for numerous or larger inflorescences, and the development of natural defenses to reduce the impact of biotic and abiotic stresses. In turn, these developments help to increase the production, survival rate, and quality of seeds produced. In addition, cereal species show a significant reduction in their ability to acclimate to low temperatures

once plants have achieved floral transition (Danyluk et al., 2003). Because of the need for a plant to achieve a sufficient level of vegetative development, a series of flowering-repression genes that prevent the expression of meristem identity genes even under promotive conditions arose to prevent early or precocious flowering in plants (Samach and Coupland, 2000; Chen et al., 1997).

The *EMF* genes (Embryonic Flower) are thought to prevent floral competence by preventing the expression of floral organ identity genes through chromatin remodeling (Moon et al., 2003). EMF2 is a homolog of the Polycomb group protein, and is thought to recruit protein complexes that transcriptionally silence the floral meristem identity genes by compacting the chromatin (Komeda, 2004; Moon et al., 2003). Once the floral promotion genes are upregulated, they are thought to outcompete the EMF-repressor complex, initiating the floral transition (Moon et al., 2003). Notably, a loss of *FCA* function (a gene from the autonomous pathway) has been shown to delay flowering even in *emf* mutants, as *emf1-1 fca-9* double mutants often did not flower, and those that did flowered later and produced more leaves than *emf1-1* mutants alone (Page et al., 1999).

The key flowering repressor in the model plant *Arabidopsis thaliana* is *FLOWERING LOCUS C (FLC)* (Michaels and Amasino, 1999; Sheldon et al., 2000b). *FLC* encodes a MADS-box protein that represses the flowering transition by blocking the expression of several meristem identity genes, notably *SOC1* and *FT* (Michaels and Amasino, 1999; Rouse et al., 2002). Until *FLC* is sufficiently and stably downregulated in the plant, inductive growth conditions will not promote flowering (Sheldon et al., 2000b; Rouse et al., 2002; Jack, 2004; Parcy, 2005). *FLC* is normally downregulated via the combined action of several independent genes classified as the autonomous pathway, but *FLC* is also responsive to vernalization treatment (Sheldon et al., 2000b; Rouse et

al., 2002; Parcy, 2005). The vernalization response is determined via a highly integrated network of gene interactions (Figure 1) (Michaels and Amasino, 1999). Certain genes such as *FRIGIDA* (*FRI*) serve to upregulate the expression of *FLC*, creating a stronger level of repression that often requires a vernalization treatment to obviate. In *FRI* mutants, the autonomous pathway is generally unable to sufficiently repress *FLC* (Shindo et al., 2005). Despite numerous efforts, as of yet there have been no FLC orthologs identified in species other than *Brassica* (Accession #AY273161, AY273162, AY273164, AY273165) and *Raphanus* (Accession #AY273160) (Genbank, http://www.ncbi.nlm.nih.gov/Genbank/).

The autonomous pathway, also referred to as the general earliness pathway, instead of functioning as a traditional linear pathway appears to instead involve a combination of synergistic gene actions that collectively respond to and maintain a favourable developmental state of the plant (Simpson et al., 2003). Once the plant has attained a suitable level of vegetative growth to support flowering, the collective expression of the autonomous pathway genes act to repress *FLC* and promote flowering through their generally independent action (Figure 1) (Blazquez et al., 2001; Sheldon et al., 2000; Michaels and Amasino, 1999). It is important to note that some genes in the autonomous pathway do work together, most notably *FCA* and *FY*, and that knockouts of any of the autonomous pathway genes lead to significant flowering delays and/or the need for a vernalization treatment (Macknight et al., 2002). The vernalization pathway can be considered as an alternative to the autonomous pathway, as it promotes flowering in plants that either have a disruption in the autonomous pathway or an aberrantly high level of repressor expression. This is likely due in part to vernalization acting to release flowering repression by *FLC*-dependant and *FLC*-independent mechanisms, while the

autonomous pathway seems to act mainly through the release of *FLC*-based repression (Michaels and Amasino, 2001). This is supported by recent evidence that suggests that the vernalization response is not solely dependant on methylation-dependent repression-based mechanisms, although reduced DNA methylation does mimic the vernalization response (Finnegan et al., 2005).

The application of gibberellic acid (GA) has been shown to accelerate flowering time in both spring and winter annual plants, but it is not a substitute for vernalization (Minorsky, 2002). Instead, gibberellic acid promotes flowering through the induction of meristem identity genes, most notably *SOC1* (Moon et al., 2003; Jack, 2004). GA treatment does not induce *FT* (Moon et al., 2003). Despite the accelerated floral transition, GA treatment does not affect *FLC* levels, instead GA action appears to operate downstream of *FLC* through the induction of flower-promoting factors rather than the release of flowering promoters (Moon et al., 2003; Sheldon et al., 2000a).

The *FCA* Gene

The *Arabidopsis* autonomous pathway gene *FCA* was initially shown to promote flowering in a study of flowering mutants (Koornneef et al., 1991). When compared to the wild-type control (*Landsberg erecta* ecotype), the *fca-1* mutation was shown to delay flowering by 17.1 days and the plant produced an additional 21.4 rosette leaves (Table 1). The single-copy *FCA* gene was mapped to *Arabidopsis* chromosome 4 (Koornneef et al., 1991; Macknight et al., 1997), and later isolated and its function characterized (Macknight et al., 1997, 2002).

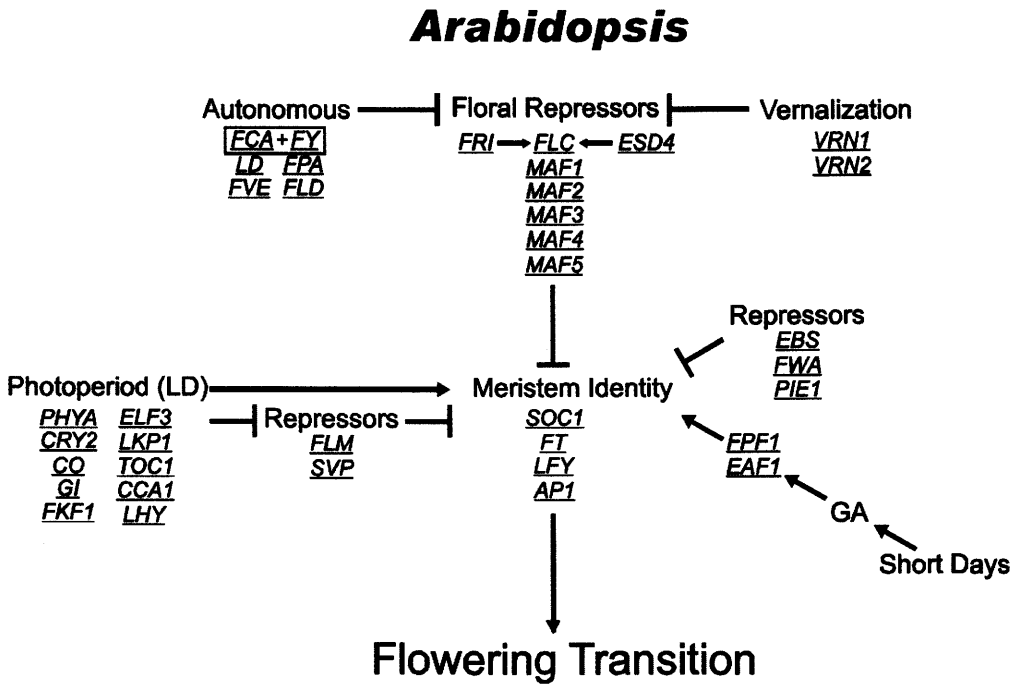

Figure 1. The *Arabidopsis* Flowering Repression Model.
Floral repressors such as FLC prevent the flowering transition even in the presence of inductive flowering conditions. Both the autonomous and vernalization pathways are able to stably downregulate these genes, allowing flowering promotion to occur.

The *fca* mutants of *Arabidopsis* have an obligate winter growth habit, with flowering delayed by approximately 3 weeks and producing approximately 15 to 20 additional rosette leaves (Table 1). A vernalization treatment is able to accelerate the floral transition in *fca* mutants, with plants flowering at a similar time as the early-flowering Ler-0 controls (Koornneef et al., 1991; Macknight et al., 1997).

There are several notable features of the *FCA* gene. *FCA* produces four different mRNA transcripts: the full-length FCA-γ, FCA-α retaining the 3rd intron, FCA-δ resulting from alternatively splicing of intron 13 resulting in a premature termination codon, and the truncated FCA-β that consists of the first 3 exons and part of intron 3 (Macknight et al., 1997). Only the FCA-γ transcript has the capacity to produce a

functional FCA protein, but the FCA-β transcript comprises the bulk of FCA transcripts (approximately 55% of the total; FCA-γ accounts for approximately 35%) (Macknight et al., 1997). The prevalence of FCA-β is due to the autoregulation of *FCA* transcripts by the FCA protein, capping its level of coding mRNA. As FCA is produced, it interacts with the FY protein to promote polyadenylation of *FCA* intron 3, resulting in the FCA-β transcript (Macknight et al., 1997; Quesada et al., 2003). FCA also requires interaction with FY to promote the flowering transition, when FCA-γ is expressed in an *fy* background, there is a significant impairment of its ability to reduce *FLC* levels (Simpson et al., 2003).

Table 1. Comparison of Flowering Delays in *fca-1* Mutants.

Study	Genotype	FLN[a] (LD)	FLN[b] (SD)	Days to flower (LD)
Koornneef et al., 1991	Ler-0 (parent)	6.8 ± 0.1	~18	27.9 ± 0.3
	fca-1	28.2 ± 0.5	~48	45.0 ± 0.7
Macknight et al., 2002	Ler-0 (parent)	5.1 ± 0.1	46.3 ± 1.4	n.d.
	fca-1	20.9 ± 0.6	>67 (n.f.)	n.d.

LD = Long Days, 16h light/8h dark. SD = Short Days, 8h light, 16h dark. n.d. = no data
a. FLN = Final Leaf Number; number of rosette leaves produced before bolting.
b. FLN for SD treatment was approximated from Koornneef et al., 1991.

FCA and *WFCA*

The *WFCA* genes isolated from wheat all share certain common characteristics. The *WFCA-A* gene contains 18 exons spanning approximately 2.6 kb, and encodes a protein 741 amino acids in length (M. Gao, T. Hoffman, A.E. Limin, D.B. Fowler, and G. Selvaraj, unpublished results). In comparison, the *FCA* gene contains 21 exons spanning approximately 2.2 kb, encoding a protein 747 amino acids in length (Macknight et al., 1997). As previously mentioned, the peptide sequence of WFCA is

highly similar to that of FCA, especially in regards to four conserved critical regions. These critical regions include the two RNA Recognition Motifs (RRMs), the WW domain (WW), and a C-terminal peptide (CTP) (Macknight et al., 1997; Sarnowski et al., 2002). Both the RRMs and WW Domain have been shown to be essential for the proper function of the FCA protein, including its self-regulation (Macknight et al., 1997; Macknight et al., 2002).

The RNA Recognition Motifs are similar to those of the ELAV protein family (embryonic lethal, abnormal vision, from Drosophila studies), and contain the characteristic RNP1 octamer and RNP2 hexamer (Figure 2) (Lisbin et al., 2000; Good, 1995; Macknight et al., 1997). While FCA lacks the third RRM common to ELAV-like proteins, the first two RRMs are hypothesized to form a single RNA-binding site (Lisbin et al., 2000). Domain replacement of the third RRM with the RRM of a related protein, either RBP9 or HuD, did not abolish ELAV protein function, while domain replacement of either of the first two RRMs resulted in largely nonfunctional proteins, especially when both RRMs were replaced (Lisbin et al., 2000).

The WW protein-protein interaction domain is not unique to FCA, and is found in various proteins in numerous species, including humans, mice, and the purple sea urchin (Simpson et al., 2003). The generalized WW domain commonly comprises approximately 40 amino acid residues and forms a globular structure containing a hydrophobic pocket (Lin et al., 2004; Schleinkofer et al., 2004). The WW domain is essential for the interaction of FCA with the FY protein, as disruption of this domain has been shown to prevent binding of FY, affecting both the self-regulatory activity of *FCA* and its role in flowering promotion (Simpson et al., 2003).

Sarnowski et al. (2002) published on the interactions of FCA with AtSWI3B, a putative homolog of SWI3 in *Saccharomyces cerevisiae*. SWI3 is one subunit of a SWI-class of ATP-dependant chromatin remodelling complexes in yeast that, although not essential for growth, impact the growth and development of numerous organisms, including humans. Using the yeast two-hybrid system, both FCA and a short FCA peptide (containing the C-terminal half of FCA) were shown to interact with AtSWI3B (Sarnowski et al., 2002). This evidence supports the hypothesis that the action of *FCA* may involve chromatin remodelling of *FLC*, as suggested by a recent study where *fca-1* plants showing altered methylation patterns of the 5' end of *FLC* were restored upon vernalization treatment (Sarnowski et al, 2002; Bastow et al., 2004).

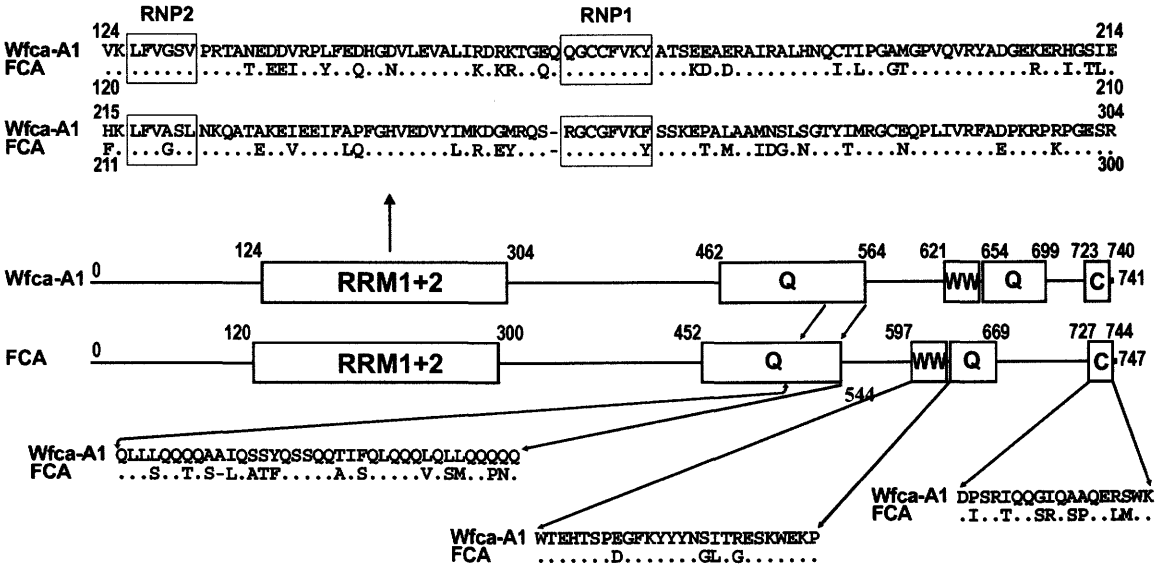

Figure 2. Comparison of FCA and WFCA Peptides.
CLUSTALW alignment of the WFCA (Gao et al., unpublished) and FCA peptides (Macknight et al., 1997) shows the similar location of the conserved domains. Amino acid locations are indicated above the peptides, and key regions have been expanded to show the alignment. Identical amino acids are indicated by dots, substitutions by the single-letter amino acid code. RRM, RNA recognition motif; RNP, RNA-binding region; Q, Q-rich region; WW, WW Domain; C, C-terminal peptide.

Classification of Genome-Associated Forms of *WFCA*.

As previously mentioned, *T. aestivum* is a hexaploid species, containing three genomes (A, B, and D). Hexaploid species commonly contain paralogous genes on each genome, and *WFCA* genes associated with each of the three genomes were classified based on the classification of specific DNA sequence variations as one of the three genomes, involving cross-genome comparisons or single-nucleotide polymorphisms, sequence insertions or deletions, and variations in tri-nucleotide repeat (TR) sequences (M. Gao, personal communication). Two genome-specific cosmid clones (A-type and D-type) were produced and sequenced, and their representative sequence variations were used as the baseline for classifying each genome type. Only those variations specific to one genome-type were used to classify the *WFCA* types. *WFCA* genes corresponding to the nucleotide variations displayed in the cosmids were selected as a representative of that genome, and plasmid stock sequences were confirmed by the variations in genome-specific restriction endonuclease cleavage patterns.

WFCA genes representing each genome were selected for use in the complementation study, and their peptide sequences were compared to determine what effect these variations would have on the peptide sequence (Figure 3). As shown in Figure 3, the D-type has two notable variations, one in the first RRM, and one in the WW domain.

The *ABAP1* Gene

Recently, the ABA-binding protein ABAP1 (Accession # AF127388) has been characterized in barley (*Hordeum vulgare*) and was obtained from an mRNA library constructed from ABA-treated barley (Razem et al., 2004). The *ABAP1* gene shares

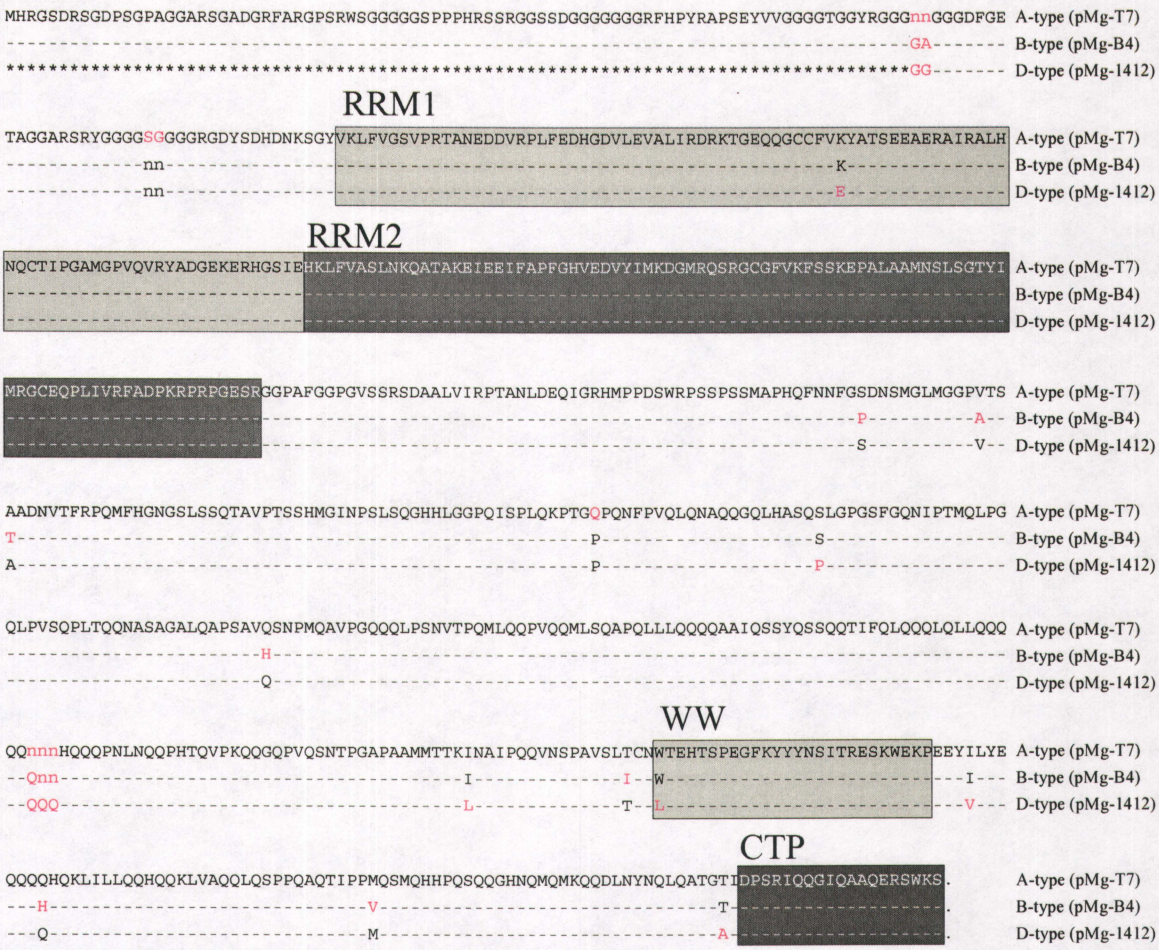

Figure 3. Variation in Genome-Associated WFCA Peptides.
Peptides of the three WFCA variants chosen for this complementation study were aligned using CLUSTALW analysis to determine genomic-specific variations. Key regions are named as in Figure 2. The A-type WFCA (pMg-T7) was chosen for the basis of this comparison. For the other two sequences, a "–" denotes a conserved residue, "*" denotes an absent residue due to the D-type partial sequence, "n" indicates an indel for that residue, and letters refer to a substitution for the corresponding amino acid. For substitutions, a red letter indicates a non-conserved variation while a black letter indicates conserved variations. The D-type WFCA sequence has two notable substitutions, one in the first RRM (K to E) and one in the WW domain (W to L).

a remarkable 95% identity with *WFCA-A* (which is 2.6 kb in length) over its entire length of 1,419 bp. In addition, the 24 nucleotides immediately preceding the designated start codon in ABAP1, as defined by Razem et al., (2004), are also present in *WFCA-A*, but starting 264 codons after the initiation codon of WFCA-A. Thus, the predicted start codon for ABAP1 corresponds to a region within the second RRM of *WFCA*.

Analysis of the peptide sequence shows that the ABAP1 peptide initiates at a methionine residue found in the second *WFCA* RRM, with the last 32 amino acid residues of the second RRM forming the beginning of the ABAP1 peptide. This suggests that ABAP1 may have arisen from a truncated form of *WFCA* or from aberrant splicing of a barley *WFCA* ortholog (Figure 4).

In addition, translating the published cDNA sequence of *ABAP1* reveals that the sequence immediately preceding the start codon (again, found in the second RRM of *WFCA*) potentially encodes 9 amino acids identical to those found in the second RRM of WFCA immediately preceding the methionine proposed as the putative ABAP1 start codon (Figure 4). In the ABAP1 study, RNA gel blots were probed for ABAP1 using ABAP1 cDNA, and in addition to the 1.8-kb band assumed to encode the ABAP1 transcript, a larger band described as being 2.6-kb in size was detected (Razem et al., 2004). The full-length *WFCA* transcript is 2.8-kb, which may correspond to the upper band in the published RNA gel blot, and suggest that there may be full-length *WFCA*-like genes expressed in barley. The size difference between the two bands (2.6-kb versus 2.8-kb) may be attributed to variations in the gene lengths that arose between wheat and barley.

To further support the possibility that *ABAP1* is related to *WFCA*, recent additions to GenBank included three barley ESTs (Accession #s BQ465154, AJ484322,

and CA026617) that when translated and aligned, show significant similarity to both the N-terminal region of ABAP1 and the corresponding region of WFCA containing the first and second RRMs (Figure 4). In both cases, the RNP domains of the RRMs are completely identical to those of WFCA. Taken together with the EST analysis, there exists a strong possibility that barley does contain *WFCA*-like genes. It is also a distinct possibility that *ABAP1* performs similar functions as the *WFCA* genes, and both gene families may be subjected to similar regulation. Whether the *ABAP1* gene, the barley *WFCA*-like genes, and *WFCA* perform similar functions is still unclear, as *ABAP1* has not been characterized in barley and WFCA has yet to be fully characterized.

Recently, it was reported that FCA, like ABAP1, is able to bind abscisic acid (ABA), and that the binding of ABA results in a disruption of FCA function (Razem et al., 2006). These authors note the strong similarity between the FCA and ABAP1 peptides, and present strong evidence that (+)-ABA is able to interact with and inhibit the activity of FCA. Specifically, when plants expressing *FCA* are treated with (+)-ABA, there is a noticeable increase in the levels of the *FCA-γ* transcript. While the *FCA-γ* transcript encodes the functional FCA protein, further analysis indicates that the transcript accumulation is due to a loss of the autoregulatory activity of FCA by preventing its association with FY. This association is assumed to occur outside of the WW domain responsible for FY binding. Previous work has shown that a modified FCA protein where the WW domain contains a loss-of-function tryptophan to phenylalanine mutation (WF) is unable to bind FY (Simpson et al., 2003). The FCA-WF protein still retains ABA-binding activity, which suggests that ABA binds to a region of the peptide

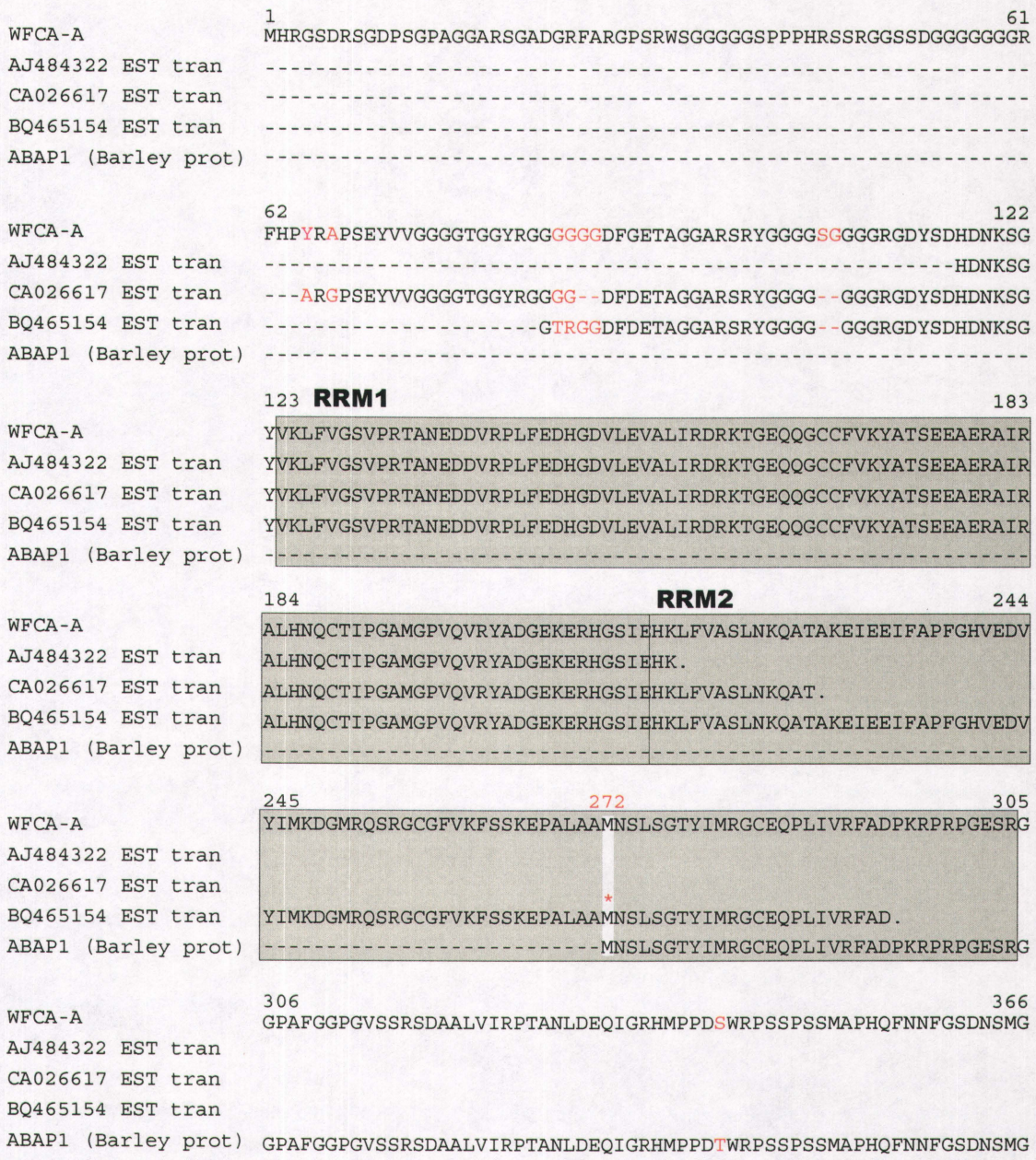

Figure 4. Alignment of Translated Barley EST Sequences with ABAP1 and WFCA-A.

The start codon for the ABA-binding protein ABAP1, as defined in Razem et al., 2004, occurs near the end of the second RNA Recognition Motif (RRM) at WFCA residue 272. An alignment of translated barley EST sequences (AJ484322, BQ465154, and CA026617) with WFCA-A and ABAP1 indicates that expressed sequences in barley contain the first and second RRM seen in WFCA. This suggests that *WFCA* genes are conserved in barley and suggests that *ABAP1* may have arisen from a truncated of *WFCA*-like gene. RRMs are designated by shaded boxes, the putative start codon for ABAP1 is designated by a red asterisk, amino acid substitutions/deletions are indicated by red letters/"-", and numbers indicate the amino acid positions of WFCA-A.

outside of the WW domain. In a future study, it would be useful to determine if WFCA has similar ABA-binding properties to ABAP1 and FCA.

MATERIALS AND METHODS

Plant Material

Arabidopsis thaliana ecotypes *fca-1* and Ler-0, originally described by Koornneef et al. (1991) were obtained from The *Arabidopsis* Biological Resource Center (Columbus, OH). The hexaploid wheat lines Norstar, Manitou, Iso 2W, Iso 4S, and Iso 5S were graciously provided by Dr. Fowler and Al Limin (University of Saskatchewan).

Arabidopsis Growth Conditions

General procedure. When *Arabidopsis* seeds were germinated on media plates, ½ MS media (2.22g MS powder (Sigma), 10g sucrose, 8g agar, water to 1 L, pH 5.8) was used. For selection of transgenic seeds on MS plates, selective ½ MS Media plates were prepared containing 50 $\mu g\ ml^{-1}$ Kanamycin. Seeds were surface sterilized in a laminar flow hood according to Clough and Bent (1998) by adding 95% ethanol and gently agitating for 1 minute. Ethanol was removed and the seeds were then agitated in a solution of 50% Javex bleach, 0.05% Tween-20 (for a final sodium hypochlorite concentration of 2.6%) for 10 minutes. Seeds were rinsed three times with autoclaved, distilled water and then placed on media plates. Seeds were distributed randomly by pipetting, or distributed in an orderly fashion by wetting sterile toothpicks and gently applying individual seeds to the surface of the media. Plates were sealed using Parafilm

(Pechiney Plastic Packaging, Menasha, WI) and placed in a Conviron growth chamber (Conviron, Controlled Environments Limited, Winnipeg, MB) under a photoperiod regime of 16h light/8 hours dark (long days) at an ambient temperature of 22°C. For germination tests, seeds were visually inspected each day, and germinated seeds were recorded at 3, 4, 5, and 6 days, with the total germinated seeds recorded. Germination was deemed successful when the radicle had visually protruded from the seed coat.

For sowing seeds in soil, seeds were thoroughly rinsed in distilled water, then transferred to autoclaved, distilled water for stratification. Stratification was used to break seed dormancy, and was accomplished by refrigerating the seeds for 3 days in a coldstream set at 4°C. After the stratification period, seeds were transferred to Rediearth (Hummert, Earth City, MO) contained in plastic trays using sterile plastic Pasteur pipettes. All plants grown in soil were placed in Conviron growth chambers (Winnipeg, MB) for growth (including germination) and vernalization. Plant growth was carried out under a photoperiod regime of 16h light/8 hours dark (long days) and temperature regime of a constant 22°C, with periodic watering and biweekly application of aqueous 20-20-20 (NPK) fertilizer (23g per 75 L water). Vernalization treatment was applied to *fca-1* plants being grown for seed production, and was generally carried out for 4-6 weeks after plants had grown to the 2-leaf stage (plant vernalization), rather than directly after sowing (seed vernalization). Vernalization chambers were set to a photoperiod regime of 8h light/16h dark under a constant temperature of 4°C after sowing into soil (as per Macknight et al., 1997).

Choice of Soil Mixture. T1 plants were grown in two different soil mixtures: Rediearth (SunGro Horticulture, Seba Beach, AB, Canada), composed of Canadian *Sphagnum* peat moss, vermiculite, a starter nutrient charge, Dolomitic limestone, and a wetting agent, or Sunshine Mix #3 (SunGro Horticulture, Seba Beach, AB, Canada), composed of Canadian *Sphagnum* peat moss, vermiculite, gypsum, Dolomitic lime, and a wetting agent. Soil mixtures were pre-wet and distributed into 72-cell trays.

Selection of Transgenic Seeds. Due to an outbreak of gnats in the phytotron, there was a severe problem with fungal contamination persisting even on sterilized T0 seeds. Initially, seeds were sterilized using the method of Clough and Bent (1998). Shortly after the seeds began to germinate, there would be a rapid and devastating growth of fungus that killed germinating seedlings. Only in some cases where the seeds were individually deposited on media plates in small numbers (<75 seeds per plate) with large intervening distances, were some plates free from contamination. However, even in the case of well-isolated seeds, there were cases where fungal growth was present. In such cases, any media displaying fungal growth was excised under sterile conditions in a laminar hood, in order to prevent it from spreading and affecting seed germination/survival. In order to screen large numbers of seeds, the standard protocol was modified to increase seed survival through a more intensive sterilization process capable of reducing the incidence of fungal outbreak.

Modifying the sterilization procedure entailed both increasing the concentration of bleach from 50% to 65% in the sterilization solution, which helped, but increasing the length of the sterilization time from 10 to 15 minutes provided a significant reduction in fungal problems. However, if seeds were suspended in water or 0.1% agarose and

simply pipetted onto the media plate, any escaping fungus would rapidly overtake the plate. Instead, if sterilized seeds were directly applied to the media plate using a wet, sterile toothpick, there was a significant reduction in the spread of fungus, and the control over seed spacing allowed for a larger space buffer to prevent fungal spread. Although this method is much more time consuming, it proved to be the most effective method of ensuring the survival of transgenic seeds during selection. Plates were then inspected every 1-2 days for the presence of fungal spots, which were promptly excised from the plate using a sterile metal spatula.

Analysis of the T1 Generation. The T0 generation was designated as the first generation of plants grown from seed collected from the transformed parent that successfully survived kanamycin selection (i.e., the first generation containing transgenic plants) (Figure 5). Seeds from the T0 generation were separated into two groups for analysis of the progeny. The first group consisted of two replicates of ~25 seeds grown on selective ½ MS media plates containing 50 $\mu g\ ml^{-1}$ Kanamycin (as for the T0 generation described above). Seed survival was used to determine the segregation of the transgene in progeny. The second group was individually seeded into separate cells of a 72-cell plastic tray alongside *fca-1* and Ler-0 controls. In total, 62 putatively transgenic seeds were sown per tray, along with 5 Ler-0 controls (early flowering control) and 5 *fca-1* controls (late flowering control). Two types of soil media were used for this study, Rediearth (Hummert, Earth City, MO) and Sunshine Mix #3 (Fisons Horticulture, Mississauga, Ontario). Two trays of each soil type were sown, with a total of 20 plants from each control (10 Ler-0 and 10 *fca-1*) and approximately 220 T1 plants

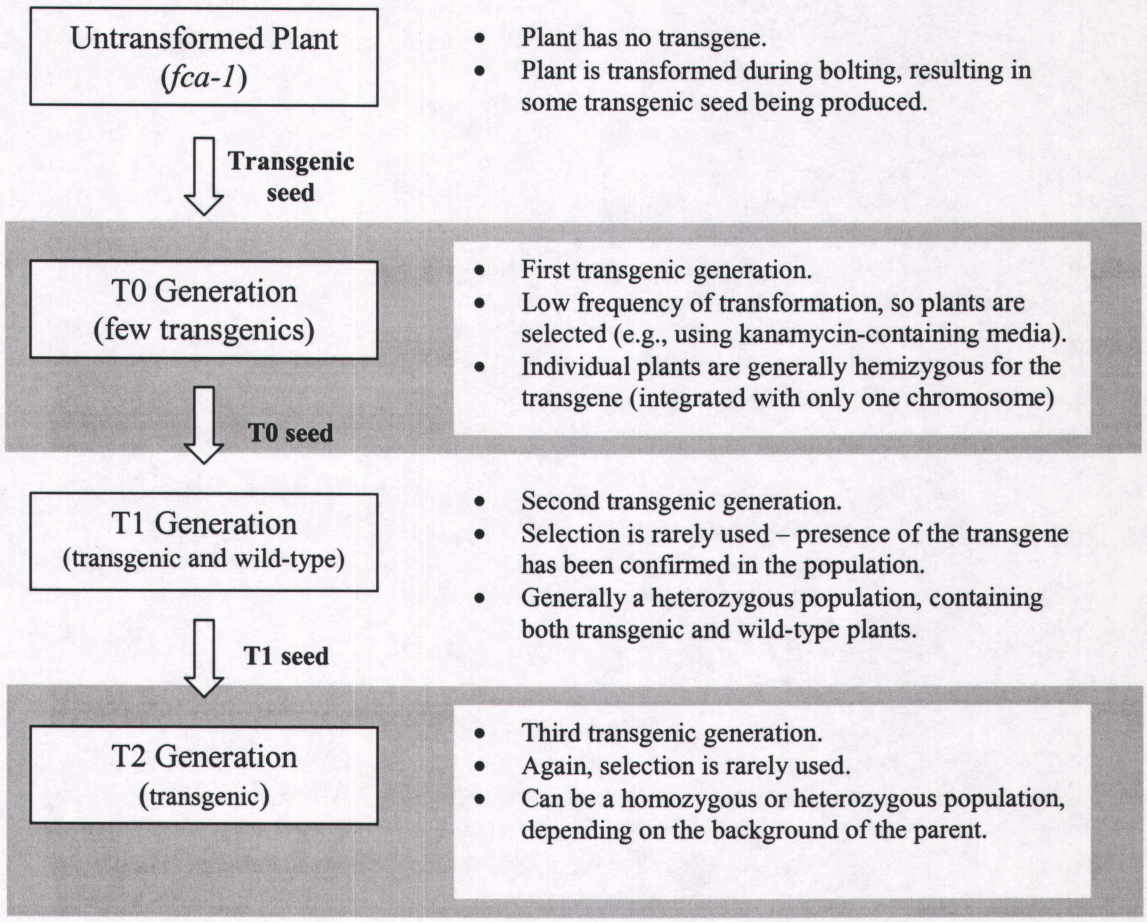

Figure 5. Explanation of Transgenic Generations.
For the purposes of this study, T0 plants are those plants initially grown from the transformed seed of the *fca-1* parent. Transgenic T0 plants are rare, and were selected for using the antibiotic kanamycin, which is detoxified in transgenic plants containing the *GUN* insertion. These plants are typically hemizygous, having the transgene integrate with only one of the homologous chromosomes. Their progeny, T1 plants, will therefore segregate with respect to the transgene, with a variable frequency depending on the number of insertions into the parental plant. T2 plants are grown from T1 seed, and these populations may be either homozygous or heterozygous depending on the status of the T1 parent. All transgenic plants are self-pollinated at all stages.

were grown in each soil mixture. This was done to minimize the chance of complications arising from the use of contaminated soil, which had previously been a problem.

Plants were grown under standard long day conditions (16 hrs light, 8 hrs dark) and their flowering behaviour compared and recorded. The FLN and bolting date was recorded and compared to the *fca-1* and Ler-0 controls. Cotyledons were included in the FLN. Once plants had bolted, rosette leaves were harvested for either GUS staining or ground under liquid nitrogen (see Materials and Methods) for use in PCR and DNA gel blot analysis.

Plant DNA Extraction

Genomic DNA extracted from wheat intended for use in DNA gel blot and RFLP analysis was isolated using the Qiagen Plant Midi Kit (Qiagen, Mississauga, ON) (described below), while wheat gDNA used for PCR amplification was purified using an SDS-based extraction (described below). Genomic DNA extracted from *Arabidopsis* plants intended for use in genotyping and PCR screening was isolated using a CTAB-buffer method (described below). Unless otherwise specified, vegetative leaf tissue was selected with a preference for younger, healthy leaves.

Qiagen Plant Midi Kit Extraction. Plant material was ground to a fine powder under liquid nitrogen in sterile, autoclaved ceramic mortars with pestles. The ground tissue was kept frozen in liquid nitrogen until transferred to a 15 mL Falcon tube and kept on ground dry ice until stored in a –80°C freezer. From this stock, approximately 1g of tissue was weighed out using clean, sterile spatulas into 2 mL microcentrifuge tubes

containing 400 µl Buffer A1 containing 100 mg ml^{-1} RNase A. The tubes were then placed in a 65°C water bath for 10 minutes with periodic inversion. 130 µl of Buffer AP2 was added, then the tube was mixed by inversion and incubated for 5 min on ice. Tubes were centrifuged 5 min at 14,000 rpm in an eppendorf 5417C microcentrifuge and the supernatant was added to a QIAshredder Spin Column. Columns were centrifuged for 2 min at 14,000 rpm and the flow-through was transferred to a clean 2-ml tube. 1.5 volumes of Buffer AP3 was added to the flow-through and mixed via repeated pipetting. 650 µl of the mixture was added to a DNeasy Mini Spin Column and centrifuged at 14,000 rpm. The flow-through was discarded and the remaining sample volume was added to the same column and centrifuged at 14,000 rpm. The column was transferred to a clean tube and 500 µl Buffer AW was added to the centre of the column. Columns were centrifuged for 1 min at 14,000 rpm, and the flow-through was discarded. The AW wash was repeated once, then the column was place in a clean, labeled tube. 100 µl of Buffer AE was pipetted into the centre of the DNeasy column, then incubated at room temperature for 15 min. Tubes were centrifuged at 14,000 rpm, then an additional 100 µl of Buffer AE was added to the column. The column was again incubated at room temperature for 15 minutes, then centrifuged at 14,000 rpm to elute the DNA. DNA quality was checked by electrophoresing 1-2 µl of the elute.

SDS Extraction. Wheat leaves were ground to a fine powder under liquid nitrogen in sterile, autoclaved ceramic mortars with pestles. Ground tissue was kept frozen in liquid nitrogen until transferred to a 15 mL Falcon tube and kept on ground dry ice until stored in a –80°C freezer. Ground material was added to 6 ml Extraction Buffer (50 mM Tris-

HCl, pH 8, 10 mM EDTA, pH 8, 100 mM NaCl, 1% SDS, 10 mM beta-mercaptoethanol) and vortexed to mix. Tubes were incubated at 65°C for 1 hour in a water bath. 2.5 ml 5M potassium acetate (pH ~4.8) was added to the tube, mixed, and left on ice for >1 hour. Tubes were centrifuged at 4,000 rpm in an eppendorf 5810R bench-top centrifuge for 30 minutes, and the supernatant (~7 ml) was filtered through Miracloth (Calbiochem, EMD Biosciences, Inc./Merck KGaA, Darmstadt, Germany) into an equal volume of isopropanol. Tubes were inverted gently to assist with precipitation, then centrifuged again at 4,000 rpm for 5 minutes to pellet the genomic DNA. Pellets were washed with 70% ethanol and air dried on the bench for 1 hour. 500 µL T.E. buffer was added to the DNA pellet, and left to resuspend overnight at 4°C. DNA was transferred to a 2-mL tube and centrifuged at maximum speed (14,000 rpm) for 30 min in an eppendorf 5417C microcentrifuge. The cleared supernatant was then transferred to a clean tube and the genomic DNA was precipitated by adding 50 µL 3M sodium acetate and 1 ml of 100% ethanol, then centrifuging the tube at 14,000 rpm for 2 min. The supernatant was removed and the pellet washed with 70% ethanol, then air-dried for 30 min on the bench. The genomic DNA pellet was finally resuspended in a total volume of 250 µl T.E. buffer. The DNA concentration was determined via UV absorbance using a spectrophotometer.

CTAB Extraction. Plant material was ground to a fine powder under liquid nitrogen in sterile, autoclaved ceramic mortars with pestles. The ground tissue was kept frozen in liquid nitrogen until transferred to a 15 mL Falcon tube and kept on ground dry ice until stored in a –80°C freezer. On a separate day, DNA was extracted by adding 5 ml of 2x

CTAB buffer (0.1M Tris-HCl, pH 8.0, 1.4M NaCl, 20 mM EDTA, 2% Hexadecyltrimethylammonium bromide (CTAB), with 50 mM β-mercaptoethanol added just before use) prewarmed to 60°C. The tubes were then placed in a 60°C water bath for a minimum of 1 hour with periodic gentle inversion to disperse any tissue clumps. DNA was extracted by adding 5 mL of a 24:1 mixture of chloroform:isoamyl alcohol and gently inverted to mix. Tubes were centrifuged at 4,000 rpm in an eppendorf 5810R bench-top centrifuge for 25 minutes, and the aqueous phase was transferred to a clean 15 mL Falcon tube. If the aqueous phase appeared to have a high concentration of secondary metabolites (e.g., green colour), it was given a second extraction with 24:1 chloroform:isoamyl alcohol. Approximately 3.3 mL (2/3 extracted volume) of chilled isopropanol (-20°C) was added to the extract and gently mixed by inversion. DNA was precipitated by centrifugation at 2,200 rpm for 10 minutes and the supernatant poured off. The DNA pellet was washed twice using 1 mL 76% ethanol in 10 mM NH_4AC (380 mL 95% ethanol, 120 mL H_2O, 0.385g ammonium acetate), gently inverted, and centrifuged at 2,200 rpm for 10 min. Pellets were air dried for 30 minutes to 1 hour and resuspended in 100 µl T.E. buffer with 10 µl RNase A, then left either overnight at 4°C or at 37°C for 30 min. DNA quality was checked by electrophoresis on an agarose gel.

Polymerase Chain Reactions (PCR)

Numerous PCR amplifications were performed throughout this study. Unless otherwise specified, all reactions were carried out in a Genius thermocycler (Techne Inc., Princeton, NJ) using the following reaction conditions:

Reagents: DNA template ~1 µl

10x PCR Buffer	10 µl
50 mM dNTP mix	1 µl
Primer #1	1 µl
Primer #2	1 µl
Taq DNA polymerase	0.5 µl
Sterile ddH_2O	36.5 µl
Final volume	50 µl

In general, 1-25 ng of DNA template (typically 10 ng) was used for plasmid amplification. For genomic PCR (wheat and *Arabidopsis*), typically 200 ng per reaction was used for amplification.

Temperature profile:

1 cycle:	94°C for 2 min.
35 cycles:	94°C for 15 seconds
	55°C for 30 seconds
	72°C for 2 min
1 cycle:	55°C for 2 min.
	72°C for 5 min
Hold:	4°C

For reactions where the annealing temperature of the primer pair was known, the annealing temperature was generally set at 3°C below the predicted annealing temperature (instead of the general 55°C) to minimize the production of non-specific amplicons.

Following PCR amplification, a portion of the reaction was electrophoresed on an agarose gel (described below) to ensure that amplicons were present, of the correct size, and as an estimate of DNA quantity.

Site-Directed Mutagenesis

In order to restore the wild-type gene sequence of the *Arabidopsis FCA* gene, site-directed mutagenesis was performed. A pair of mutagenic primers containing the desired nucleotide sequence was used to amplify the mutant gene in a standard PCR reaction. Following PCR amplification, the reaction was digested with DpnI for 2 hours, then electroporated into electrocompetent XL1-Blue cells for selection. As DpnI only cleaves methylated DNA, it will only cleave the original plasmids, as the new molecules

were synthesized in a methylase-free reaction. Plasmid DNA was isolated from surviving colonies and sequenced to confirm the desired mutation. The corrected FCA-γ gene and pTH30-10 were digested with BsmBI at 55°C for 2 hours, then both reactions were electrophoresed to separate the DNA fragments. The 4.5 kb fragment from pTH30-10 and 799 bp fragment from the mutagenized FCA-γ were excised and purified for ligation. The corrected FCA-γ insert was ligated into the pTH30-10 backbone and electroporated into XL1-Blue. Colonies were screened by isolating their plasmids and screening them to determine if they contained the insert in the correct orientation by restriction digestion with BamHI. DNA was prepared from cultures containing the appropriate insert orientation and sent for sequencing to confirm the FCA-γ sequence.

Plasmids and Vectors

The pBluescript II SK(+) vector (Stratagene, San Diego, CA), *WFCA*-containing plasmids pMG-T7, pMG-T5, pMG-B4, and the D-type *WFCA* cDNA-containing plasmid pMG-1413A1 were provided by Dr. Gao (NRC PBI). The binary vectors RD410, RD412, pHS990, and pHS737 were provided by Rozina Hirji (NRC PBI). Protein expression vector pET-28a was purchased from Novagen (EMD Biosciences, Inc./ Merck KGaA, Darmstadt, Germany). Vectors pTH-31 to pTH-35 and pMg-B6-MB were produced for this study (described below).

Table 2. Plasmids Obtained/Constructed for Use in This Study.

Plasmid Name	Backbone	Insertion	5'-restriction	3'-restriction
pMg-T7	pBS II SK+	*WFCA-A1*	BamHI	EcoRI
pMg-B4	pBS II SK+	*WFCA-B2*	BamHI	EcoRI
pMg-1413A1	pBS II SK+	*partial WFCA-D*	BamHI	EcoRI
pMg-DA4	pBS II SK+	*WFCA-D hybrid*	BamHI	EcoRI
pHS737	pHS737	MCS	BamHI	EcoRI
pTH-31	pHS737	*WFCA-A1*	BamHI	EcoRI
pTH-32	pHS737	*WFCA-B2*	BamHI	EcoRI
pTH-33	pHS737	*WFCA-D hybrid*	BamHI	EcoRI
pTH-34	pHS737	*FCA-γ* (mutant)	BamHI	EcoRI
pTH-35	pHS737	*FCA-γ* (restored)	BamHI	EcoRI
pMg-B6-MB	pET-28a	*WFCA-B2*	BamHI	none (blunt)

Digestion of DNA Using Restriction Endonucleases

Restriction digestion of DNA was generally performed using enzymes purchased from Invitrogen (Carlsbad, CA) or New England Biolabs (Beverly, MA). With the exception of genomic DNA digests, reaction volumes were kept small so as to facilitate loading on agarose gels. Typical volumes ranged from 10 – 20 µl final volume.

DNA digestions were typically prepared by combining the DNA, 1/10 the total volume of the required 10x buffer (as specified by the supplier), 1/10 the total volume of bovine serum albumen (BSA) only if required, and approximately 2-10 units of the restriction endonuclease, depending on the length of digestion and the activity of the enzyme. Digestions were briefly vortexed at low speeds and given a brief centrifugation to collect the reaction at the bottom of the reaction tube. Unless otherwise specified, all reactions proceeded for a minimum of 1 h in a 37°C incubator. Digestions requiring variant temperatures were incubated in a water bath set to the appropriate temperature.

For procedures involving digestion with the same endonuclease, such as the preparation of multiple inserts for cloning or screening of plasmid preparations, a master mix was prepared containing water, the required buffer, 1x BSA if required, and the restriction endonuclease. This mix was then pipetted into separate reaction tubes, followed by the addition of the target DNA.

DNA Ligation

All DNA ligations were performed using T4 DNA ligase purchased from Invitrogen (Invitrogen, Carlsbad, CA), and incubated for a minimum of 12 hours in a 16°C water bath. Ligations typically involved a 5:1 molar excess of the insert to the plasmid backbone. All ligations were performed in a final volume of 25 µl.

Bacterial Strains

Escherichia coli (*E. coli*) strains DH5α (Bethesda Research Laboratory, Invitrogen, Carlsbad, CA) and *Agrobacterium tumefaciens* strain GV3101 containing plasmid pMP90RK (Koncz and Schell, 1986) were obtained from Rozina Hirji (NRC PBI). The *E. coli* strain XL1-Blue (Stratagene, San Diego, CA) was obtained from Dr. Ming Gao (NRC PBI). *E. coli* strains BL21, BL21(DE3), and BL21 (pLysS) were purchased from Novagen (EMD Biosciences, Inc./Merck KGaA, Darmstadt, Germany).

Culture Conditions

Unless otherwise specified, all cultures were grown in autoclaved Luria-Bertani broth (1 litre: 10g tryptone (Bacto Laboratories Pty Ltd, Liverpool, NSW, Australia), 5g

yeast extract (Bacto Laboratories Pty Ltd, Liverpool, NSW, Australia), 10g NaCl, pH 7.0). For selective cultures, antibiotic stocks were added to a master stock directly beside a lit bunsen burner. For liquid stocks, ampicillin was added to a final concentration of 100 $\mu g\ ml^{-1}$ and kanamycin was added to a final concentration of 50 $\mu g\ ml^{-1}$.

Unless otherwise specified, all culture media plates were produced by adding 15g agar/L Luria-Bertani broth, autoclaving for 30 minutes under a liquid cycle, and left to stir at room temperature until the flask had cooled to ~55°C. If antibiotic selection was required, antibiotics were left at room temperature until completely thawed, and added to the culture either in a laminar flow hood or directly beside a lit Bunsen burner. Ampicillin was added to a final concentration of 100 $\mu g\ ml^{-1}$ and kanamycin was added to a final concentration of 50 $\mu g\ ml^{-1}$. The media was then stirred for a minimum of 10 minutes to allow for even distribution of antibiotic in the solution. Approximately 30 ml of media was poured into sterile 100 mm x 15 mm petri dishes (Fisher Scientific, Pittsburgh, PA) either in a laminar flow hood or directly beside a lit bunsen burner, swirled twice to distribute any air bubbles to the side of the plate, and then left to solidify either in the hood or on the bench. Plates were generally allowed to dry on the bench for approximately 3 days to remove excess moisture, or if the pates were used immediately they were dried for 15-20 min in a laminar flow hood with approximately half of the lid open. All media plates were stored in the original packaging at 4°C in the Coldstream, with excess air removed and taped shut using time tape. All bags were clearly labeled with the type of selection and date made.

Unless otherwise specified, all bacterial liquid cultures were prepared by inoculating 4 ml of LB media containing the appropriate antibiotic with either a

randomly picked colony or 3-5 µl of a starter culture. *E. coli* cultures were grown at 37°C for 12-16 hours with mild agitation (~250 rpm), while *Agrobacterium tumefaciens* cultures were typically grown for 1-2 days at 28°C with mild agitation (~220 rpm). Glycerol stocks were prepared by adding 750-1000 µl of the culture to an equal amount of sterile 65% glycerol in a Cryovial (Nalge Nunc International, Rochester, NY). Stocks were mixed by pipetting and immediately frozen in liquid nitrogen. All glycerol stocks were stored at –80°C in an upright freezer.

Preparation of Electrocompetent Cells

Electrocompetent cells were prepared for use in bacterial transformation via electroporation. 5 ml selective cultures (*E. coli* strains XL1-Blue, BL21, BL21 (DE3), and BL21 (pLysS); and the *Agrobacterium tumefaciens* strain GV3101) were grown for 12-16 hours at 37 °C (for *E. coli*) or 28°C (for GV3101), then used to inoculate 500 ml LB media and grown overnight. Cultures were grown to an OD_{600} of 0.5-0.7 (early- to mid-log phase), then chilled on ice for a minimum of 30 min. The culture was transferred to chilled centrifuge bottles and centrifuged at 4000xg for 15 min at 4°C. Supernatant was removed and the cells were resuspended in 500 ml ice-chilled 10% glycerol (presterilized). The resuspended cultures were centrifuged a second time (as before) and resuspended in a volume of 250 ml. This step was repeated, resuspending the cells in 25 ml, and finally in 2 ml 10% glycerol. Between centrifugations, the cells were kept on ice. From the 2 ml suspension, cells were aliquoted into sterile centrifuge tubes in 50 µl, 100 µl, and 200 µl volumes and immediately frozen in liquid nitrogen. Competent cell stocks were kept frozen at –80°C until needed.

DNA Transformation

For electroporation of plasmids, the DNA suspension was dialyzed on 0.05 μm nitrocellulose MF-Millipore membrane filters (Millipore, Bedford, MA) floating in T.E. buffer for 30 minutes to remove excess salts, if needed. For all reactions, 1 mm cuvettes were used and the electroporator was set at 200 Ω. For transformation into all *E. coli* strains, a charging voltage of 1.80 kV was used. For transformation into GV3101 cells (*Agrobacterium*) a charging voltage of 1.44 kV was used.

Electrocompetent cells were thawed and cuvettes were chilled on ice for approximately 10 minutes. In general, 1-2 μl of the DNA suspension was added per reaction (50 μl competent cells) and gently mixed by flicking the side of the tube with a finger. The cells were pipetted into the cuvette, which was then tapped to remove any air bubbles and any external condensation wiped off. Cuvettes were placed in the BioRad Gene Pulser electroporator (BioRad, Hercules, CA) and pulsed. After pulsing, the time constant was checked, and ranged from 4.1-4.6 ms for successful transformations. The cuvette was quickly removed from the electroporator and 1 ml of LB media was pipetted to resuspend the cells, then transferred to a 15 ml Falcon tube and incubated for 1 hour at 37 °C (30°C if GV3101) with gentle agitation. After incubation, aliquots of 25, 125, and 250 μl were spread on selective plates and left to grow at 37 °C (30°C if GV3101) overnight. Surviving colonies were picked and their cultures screened to confirm successful insertion of the plasmid.

Agarose Gel Electrophoresis

Before electrophoresis, DNA samples were mixed with 1/6 volume 6X type II DNA Loading Buffer (15% Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol), followed by a brief centrifugation to collect the sample at the bottom of the tube. Depending on the size of the DNA fragments being resolved, one or both of two DNA ladders was used for size comparison. The λ/HindIII ladder (fragment sizes 23130, 9416, 6557, 4361, 2322, 2027, and 564 bp) was used to estimate DNA fragment sizes between 1 kb and 23 kb, while ϕX174/HaeIII (fragment sizes 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, and 72 bp) was used to estimate the sizes of DNA fragments less than 1.5 kb. Both ladders were produced and provided by Rozina Hirji (NRC PBI).

Typical agarose gels were 0.8%, prepared by adding 8 g/L agarose to 1x TAE buffer (40 mM Tris acetate, 2 mM EDTA, pH ~8.5). The solution was then microwaved until all traces of agarose had gone into solution as evidenced by visual inspection. The gel solution was then cooled to 65°C and 0.5 μg ml^{-1} ethidium bromide was added to the gel solution. For gels requiring a different concentration of agarose, the amount of agarose added in the initial step was varied accordingly (e.g., 1g in 100 ml for a 1% gel). Gels were cast using the Horizon 11-14 or Horizon 58 apparatus (Life Technologies (Gibco BRL)/Invitrogen, Carlsbad, CA), and allowed to solidify for a minimum of 20 minutes before use.

Prior to loading, agarose gels were covered with 1x TAE buffer until the solution just covered the upper surface of the gel. For those gels used to separate DNA fragments that were to be isolated, the entire running apparatus was drained and rinsed 3 times with distilled water before adding the gel. Electrodes were connected to the running apparatus

and current was supplied by a Whatman/Biometra Model 250 power pack (Whatman/Biometra, Goettingen, Germany). Gels were typically run at 60-90 V for 30-90 minutes depending on the degree of separation required. Certain gels were examined during running by excitation at 365 nm using a handheld UV lamp (UVP model UVL-28, Upland, CA). Upon sufficient resolution of the DNA fragments, the gel was removed from the running apparatus, blotted at its ends using paper towel to remove excess TAE buffer, and visualized using a Foto/Analyst Visionary system (Fotodyne, Hartland, WI).

Gel Purification of DNA Fragments

Unless otherwise noted, all DNA fragments were purified from agarose gels using the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON). The bands were visualized on a transilluminator covered with a plastic cover that was washed 3x prior to use. The agarose gel was placed on a suitably-sized piece of Saran Wrap to minimize contamination from the transilluminator. Bands were quickly excised using a new, clean #11 scalpel blade and transferred to a labeled 2 mL tube, and weighed using an analytical balance to determine the weight of the gel slice. Volume was estimated with each 100 mg gel equivalent to approximately 100 µl.

Three volumes of buffer QG was added to the gel slice, then incubated at 50°C for a minimum of 10 minutes with occasional inversion, until the gel slice had been completely solubilized as determined by visual inspection. For DNA fragments less than 500 nt or over 3.8 kb in length, 1 volume of isopropanol was added to the solubilized gel fragment and vortexed briefly. The sample was transferred to a QIAquick spin column by pipetting the sample into the centre of the column and centrifuged at top speed

(14,000 rpm) in an eppendorf 5417C microcentrifuge for 1 min. The flow-through fraction was discarded, and if the sample was involved in blunt-end ligation, 0.5 mL of buffer QG was added and the column centrifuged at top speed for 1 minute. 0.75 mL of wash buffer was then added to the column, let stand for approximately 3 minutes, then centrifuged at top speed for 1 min. The flow-through was discarded and the column again centrifuged at top speed for 1 min. to remove any remaining ethanol from the column. The cartridge was then placed in a clean, labeled 1.5-mL microcentrifuge tube. For DNA fragments used for ligation, 30 µl of elution buffer EB (10 mM Tris-HCl, pH 8.5) was added to the centre of the column, for all others 50 µl of elution buffer EB was added. Columns were let stand for 1 min., then centrifuged at top speed to elute the DNA fragment. The column and tube were checked to make sure that the correct sample was collected and the cartridge discarded. DNA not used immediately was stored at either 4°C or 0°C until used.

Production of Binary Vectors

Binary vectors pTH31, pTH32, and pTH33 were produced by digesting pMG-T7, pMG-B4, pMG-DA4, and pHS737 with the restriction endonuclease KpnI for 2 hours at 37°C. Following digestion, the reaction was stopped by heat-killing the endonuclease by incubation at 75°C for 15 minutes. 1/10 volume of 3M sodium acetate (pH 5.2) was added to the reaction and mixed, followed by the addition of 2.5 volumes of 95% ethanol (e.g., for a 20 µl reaction, 2 µl of 3M sodium acetate and 55 µl of ethanol would be added). The reaction was then mixed and allowed to precipitate at –80°C for a minimum of 1 hour. The reaction was centrifuged at 14,000 rpm for 30

minutes and the supernatant removed. The tube was washed with 70% ethanol and centrifuged at 14,000 rpm for 10 minutes to remove excess salt from the DNA pellet. The supernatant was removed and the DNA dried in a speedvac under vacuum for approximately 15 minutes. The DNA was resuspended in approximately 10 µl T.E. buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and mixed. The DNA was then digested a second time in a final volume of 15 µl with BamHI for 2 h. The reaction was heat-killed and precipitated as above. The endonuclease PvuI was used to digest the pBS backbone to better separate the *WFCA* fragment due to the small size difference between the bands (~2.8 kb *WFCA* vs. 3.0 kb pBS). The resuspended Bam/Kpn digestions were digested with PvuI for 3 h, heat killed as above, then electrophoresed on an agarose gel.

Binary vectors pTH34 (mutant *AtFCA*) and pTH35 (SDM-corrected *AtFCA*) were constructed by digesting pTH30-10 (mutant *AtFCA*) or pTH30-10R (SDM-corrected *AtFCA*) with KpnI for 2 hours at 37°C, then heat-killing the enzyme and ethanol precipitating the reaction as above. Following resuspension in T.E. buffer, each reaction was restriction digested a second time with EcoRI for 2 hours at 37°C, followed by the immediate addition of DNA loading buffer and electrophoresis of the reaction on a 0.8% agarose gel. The full-length *FCA*-containing fragment (2.3 kb) was isolated from both pTH30-10 and pTH30-10R and the pHS737 backbone (13.7 kb) were purified using the QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON).

All *WFCA/FCA* inserts were ligated into the pHS737 binary vector backbone using the standard DNA ligation procedure described earlier, transformed into the *E. coli* strain XL1-Blue, and selected for Kanamycin resistance. Resistant colonies were selected, DNA isolated and sequenced to confirm successful insertion of the target gene,

and then transformed into the *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986) using the procedure described earlier.

Expression of WFCA Protein

Construction of the expression plasmid. In order to raise antibodies against WFCA (see Appendix 2), plasmid pMG-B6 containing the *WFCA-A2* gene (AY#230399) was cloned into the pET-28a. In order to clone the *WFCA* gene in-frame, there was a small 5' section (108 bp in length) of the cloned *WFCA-A2* gene that had to be deleted. This deletion ended at the start codon (atg), resulting in the cac triplet encoding the first peptide residue (H) being located just after the ggatcc nucleotide sequence denoting the BamHI restriction site in pET-28a.

pMG-B6 (containing *WFCA-A2*) was digested using SmaI for 3 hours in a 30°C water bath to linearize the plasmid, then heat-killed at 75°C for 15 min. The reaction was ethanol precipitated to remove the restriction endonuclease and buffer salts, and the resuspended plasmid was then divided into two tubes for use in two separate reactions.

One tube of the SmaI-linearized pMG-B6 reaction was further digested with NspI for 2 hours and electrophoresed on a 0.8% agarose gel. The 1,011 bp NspI/NspI fragment was gel purified. Following gel purification, the NspI/NspI fragment eluted in 30 µl of water. The NspI/NspI fragment was blunt ended using the Klenow fragment of DNA Polymerase I by adding 4 µl 10x EcoPol Buffer, 5 µl 10 mM dNTP mix (final concentration 33 µM each dNTP), and 1 µl Klenow Fragment of DNA Polymerase I (New England Biolabs, Beverly, MA) (final volume 40 µl). The reaction was allowed to proceed at room temperature for 15 min, then stopped by adding 0.8 µl 0.5M EDTA

(final concentration ~10 mM EDTA). The blunt-ended fragment was ethanol precipitated for 1 hour, then resuspended in 10 µl T.E. buffer. Finally, the fragment was digested with BsmBI for 2 h in a 55°C water bath, then loading dye was added and the reaction was immediately electrophoresed. The 265 bp NspI/BsmBI fragment was isolated and gel purified, and saved for use in ligation later on.

The second tube containing the SmaI-linearized pMG-B6 was digested using BsmBI for 2 h in a 30°C water bath. The reaction was then electrophoresed and the 5kb BsmBI/SmaI fragment was isolated and gel purified. This fragment was used as the backbone for ligation with the NspI/BsmBI insert.

The Nsp/BsmBI and BsmBI/SmaI pMG-B6 fragments were ligated together using the conditions outlined above, with the NspI/BsmBI insert added in approximately 5x excess of the BsmBI/SmaI backbone. The ligation was allowed to proceed overnight in a 16°C water bath, and was dialyzed for 30 min on T.E. buffer to remove excess salts. 2 µl of the dialyzed ligation reaction was electroporated into XL1-Blue *E. coli* cells for selection on Ampicillin media. Resistant XL1-Blue colonies were picked and cultured as described above, plasmids purified using the Qiagen Plasmid Mini Kit (Qiagen, Mississauga, ON), and sent for sequencing. Additionally, plasmids were screened by SpeI digestion to ensure replacement of the 1,078 bp fragment of pMG-B6 with the modified 960 bp fragment (containing the 5'-end deletion).

Once the replacement was confirmed, plasmid pMG-B6-MB (so named as to keep the source plasmid indicated) was digested with KpnI for 1 h. Expression vector pET-28a was digested with HindIII for 1 h. Both plasmids were then blunt-ended using the Klenow fragment as described above, then gel purified. The purified plasmids were

then digested with BamHI for 1 hour and gel purified. The pMG-B6-MB insert was ligated to the pET-28a backbone in an approximately 5:1 ratio of insert:backbone as above. The ligation mixture was transformed into electrocompetent XL1-Blue cells to maintain the plasmid, and sequenced. Upon verification of the plasmid sequence, pMg-B6-EX was transformed into electrocompetent BL21, BL21 (DE3), and BL21 (pLysS) cells. DNA was prepared from cultures from each cell line and screened using a BamHI/XhoI double-digest. Cell cultures containing the appropriate plasmid were used to prepare glycerol stocks and for expression of WFCA.

Protein induction. Initially, when pMG-B6-EX2 transformed BL21 (DE3) cells were grown in selective culture and induced with 1 mM IPTG (pET-28a contains the T7*lac* promoter) for 2 hours, there was no significant induction of protein. To test the stability of the plasmid, BL21 (DE3) and BL21 (pLysS) strains containing pMG-B6-EX2 were spread on a series of plates: non-selective LB plates, selective LB-Kan plates, non-selective LB plates with 1 mM IPTG, and selective LB-Kan plates with 1 mM IPTG. Colonies were formed only those plates lacking IPTG, and therefore lacked protein expression. Because WFCA appeared to be toxic to the *E. coli* cells, a low-temperature induction protocol was tried.

When induced cultures were grown at lower temperatures (30°C, 26°C, and 24°C), there was some protein expressed in the 26°C and 24°C cultures, which was then shown to be localized to the insoluble fraction (inclusion bodies). Culture conditions were further modified after the methods described in Blackwell and Horgan (1991). Based on their report, the inclusion of sorbitol and betaine in culture media was able to substantially improve the production of active protein, and was hypothesized to improve

the recovery of other recombinant proteins. When pMG-B6-EX2 was expressed in media containing 0.66M-1M sorbitol and 2.5 mM betaine, a small amount of theWFCA protein was found in the soluble fraction, but there was also a significant improvement in the levels of protein found in the insoluble fraction. As the levels of soluble protein were quite low, protein was isolated from the insoluble fraction of induced BL21 (DE3) cells grown at 25°C in LB media supplemented with 0.66M sorbitol and 2.5 mM betaine.

Protein extraction. Culture was dispensed into pre-weighed centrifuge bottles, then centrifuged at 10,000g for 10 min. Supernatant was decanted and the weight of cell residue was recorded. Cultures was resuspended in 40 ml 1x Binding Buffer (0.5M NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9). 1.4mg g^{-1} cell residue of lysozyme was added to resuspended cells and incubated at 30°C for 15 min. Following lysis, cultures were placed on ice and sonicated using a 3 mm microtip with power setting of 5 on a Sonicator XL2020 (HeatSystems, Farmingdale, NY). Each sonication comprised 20 pulses with a 30s cooling time between pulses. Sonicated cells were centrifuged at 5,000g for 15 min and resuspended in 20 ml 1x Binding Buffer. Cells were sonicated again as before, centrifuged at 5,000g for 15 min, and resuspended in 5 ml 1x Binding Buffer with 6M urea. Cell extracts were incubated on ice 1 h, then centrifuged at 16,000g for 30 min. Supernatant was filtered with a 0.45 micron filter.

Quick 900 cartridges (Novagen, EMD Biosciences, Inc./Merck KGaA, Darmstadt, Germany) were equilibrated with 6 ml 1x Binding Buffer w/ 6M urea. Cell extracts were then passed through cartridges using a sterile disposable syringe. Cartridges were washed with 20 ml 1x Binding Buffer w/ 6M urea followed by 10 ml 1x Washing Buffer w/ 6M urea (0.5M NaCl, 20 mM imidazole, 20 mM Tris-HCl, 6M urea,

pH 7.9). Protein was eluted from columns using 4 ml 1x Elution Buffer w/ 6M urea (1M imidazole, 0.5M NaCl, 20 mM Tris-HCl, 6M urea, pH 7.9), and stored at 0°C until separated using SDS-PAGE.

Agrobacterium-Mediated Transformation

This procedure was based on the method of Clough and Bent (1998). *Agrobacterium tumefaciens* strain GV3101 was used for all plant transformations (Koncz and Schell, 1986). Binary vectors were transformed into electrocompetent GV3101 cells using the protocol from the Uppsala Transgenic *Arabidopsis* Facility, with the exception that electrocompetent GV3101 cells were prepared using sterile 10% glycerol in place of 1 mM Hepes buffer. For electroporation, a BioRad Gene Pulser set to 25μFD capacitance with the BioRad Pulse Controller Plus set to a resistance of 190 ohms was used. GV3101 cells were thawed on ice for 10 minute prior to transformation, and 1 mm cuvettes were chilled on ice for the same period. Approximately 100 ng of DNA was used in a final volume of 1-2 μl for each transformation, and was prepared by dilution of the plasmid in sterile water. DNA was added to individual tubes, mixed by flicking the bottom of the tube with a finger to mix, then transferring the cells to a 1 mm cuvette. Excess condensation was removed by wiping the surface of the cuvette with a Kimwipe, and any air bubbles in the cell solution were removed by tapping the cuvette on the bench until none were visible. Cuvettes were pulsed for approximately 5 ms at 1.44 kV and immediately resuspended in 1 ml of LB media via pipetting the mixture up and down within the cuvette. The resuspended cells were transferred to a 15 ml Falcon tube and incubated at 28°C with mild agitation (approximately 200 rpm) for 1 hour.

Following this regeneration period, the *Agrobacterium* culture was plated on selective LB media plates and incubated at 28°C for 2 days.

Colonies appearing on the selective plates were randomly picked using sterile toothpicks and used to inoculate selective 4 ml LB cultures. Cultures were grown at 28°C with mild agitation (approximately 220 rpm) for 1-2 days and a sample was taken for screening for the binary vector using restriction endonuclease digestion. Successfully transformed GV3101 cultures were used to prepare glycerol stocks as mentioned previously, and the remainder of the culture was then prepared using the Qiagen Miniprep Kit (Qiagen, Mississauga, ON) and the plasmid sequenced to ensure the correct insertion.

fca-1 plants intended for use in transformation with *Agrobacterium tumefaciens* strain GV3101 were prepared by germinating seeds in soil as previously mentioned, except that after transferring the stratified seeds to soil in 3.5" square plastic pots (3.5" x 3.5" x 3.5"), the pot was covered with a fine rubber mesh secured with an elastic band. Plants were grown as above and vernalized for 30 days once the plants reached the two-leaf stage. After the plants began to bolt, the bolts were severed at the base of the plant using a clean scalpel blade. Once the secondary bolts developed, the plant was then dipped into the Agrobacterium suspension, described below.

Transformation of plant material was accomplished by streaking *Agrobacterium tumefaciens* colonies containing the appropriate binary vector from prepared glycerol stocks onto selective plates and incubating for approximately 3 days (Clough and Bent, 1998). Cultures were used promptly to ensure that no recombination took place before the plasmid was used for transformation. Colonies were randomly picked and used to inoculate 2-3 6 ml cultures containing 50 $\mu g\ ml^{-1}$ Kanamycin. Cultures were incubated

at 30°C with gentle agitation for a minimum of 12 h. 5 ml of turbid cultures were used to inoculate a 2L Erlenmeyer flask containing 500 ml LB media (without Kanamycin). The remainder of the culture was screened using either PCR or restriction digestion (as outlined previously) to ensure the vector was present in the Agrobacterium culture. Culture-containing flasks were incubated at 30°C with gentle agitation until the media reached an OD_{600} of at least 0.7, then dispensed into sterile, autoclaved centrifuge bottles and centrifuged at 6,000xg at 4°C for 30 minutes. The cells were resuspended in a 5% sucrose solution containing 0.05% Silwet L-77 surfactant (OSi Specialties, Inc., Greenwich, CT) to an approximate OD_{600} of 0.9. Plants were then hand dipped in the *Agrobacterium tumefaciens* mixture with gentle swirling for 3-5 seconds, then removed and placed on their side in a plastic tray and covered with a transparent dome to maintain humidity. Approximately 60 plants were used for transformation with each construct. Dipped plants were placed in a dark room at room temperature for 24 hours, then placed upright in a isolated growth chamber and covered with plastic sleeves to assist with seed harvest. Plants were grown to maturity under LD conditions (16 hrs light, 8 hrs dark) with a constant temperature of 22°C. Seeds were harvested using standard procedures (described above) and segregated for individual plant progeny, with care taken not to release transgenic seed. All transgenic seeds were sieved at harvest to remove debris, clearly labelled, and stored in either sterile eppendorf tubes or manila envelopes.

Screening of Transformed Plants

Seeds from *Agrobacterium*-transformed plants were screened for the T-DNA insert by plating the seeds on ½ MS plates containing 50 μg ml^{-1} Kanamycin. The T-

DNA insertion contains the NPTII gene conferring resistance to Kanamycin, so only those plants that express the T-DNA cassette should survive on the selective media. Seeds were sterilized and plated as above, then the surviving, green seedlings were transferred to soil once they reached the 4-leaf stage. Surviving plants were gently extracted from the media plate using sterile forceps, gently rinsed in a dish containing sterile water for 2 minutes to remove residual media and antibiotic, and placed either in a small pot or plastic flats containing moistened Rediearth media. The roots were gently covered with soil and the pot was grown under standard conditions (described above) in an isolated growth chamber. No vernalization treatment was given. As these plants were transgenic, they were covered with aracons, hollow plastic cylinders that isolate neighbouring plants to prevent cross fertilization. Plants were grown to maturity and seed was harvested using 0.5 mm sieve screens (equivalent to Tyler #32 mesh) (Fisher Scientific, Pittsburg, PA).

DNA Gel Blot Analysis

Genomic DNA used for DNA gel blot analysis was extracted using the Qiagen Plant Mini Kit according to the protocol described above. All genomic DNA used for DNA gel blot analysis was digested with the appropriate restriction endonucleases for a minimum of 5 hours, and in cases where different probes were being hybridized to the same digestion treatments, in order to minimize variation in the amount of DNA used, a single digestion reaction was performed and then divided for use in each hybridization.

Digested genomic DNA samples were run on a 0.6% agarose gel containing 50 ng μl^{-1} ethidium bromide, photographed under UV light, and then blotted onto Hybond-

N+ membrane according to the following procedure: Agarose gels were pre-treated by rinsing with distilled water, followed by depurination in 0.125M HCl for 10 min with gentle agitation. Gels were rinsed for 2 min in distilled water, denatured for 30 min with gentle agitation in a 1.5 M NaCl, 0.5 M NaOH solution, then rinsed for 2 min in distilled water, followed by neutralization in 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.5) for 30 min with gentle agitation. Gels were given a final distilled water rinse and vertically blotted using 20x SSC as a transfer buffer. Gel transfer was carried out for a minimum of 24 h, followed by cross-linking of the membrane using a UV Stratalinker (auto crosslink setting) before rinsing in 2x SSC. Membranes were stored in Saran Wrap at 4°C until used for hybridization.

Hybridization was carried out using 2 different *WFCA*-specific probes, a 150-bp WFCA probe (specific to exon 13) or the HindIII/XhoI fragment of Iso2W-1e8 (1,355 bp spanning part of intron 10 to exon 14). Membranes were pretreated by incubation in 25 ml of Hybridization Buffer (0.5M Na_2HPO_4 pH 7.2, 7% SDS) at 65°C for at least 1 hour.

The short probe was radioactively labeled using a Klenow fragment-based reaction, with DNA diluted to a final concentration of ~1 μg and added to a reaction tube containing 1 μl 5 mM dNTP mix (dTTP and dGTP) was added. 10 μCi P^{32}-dATP and 10 μCi P^{32}-dCTP was added to the reaction, followed by 1 U of Klenow fragment. The reaction was allowed to proceed for 15 min at room temperature, then stopped by the addition of 1 μl 0.5 M EDTA. Probes were then purified using a NICK column with a final elution volume of 400 μl and cpm was detected.

The large probe was prepared by diluting 25 ng of the gel purified fragment in 45 µl T.E. buffer and following the Rediprime II (Amersham Pharmacia) random labeling protocol. The probe was purified using a NICK column (Amersham Pharmacia) in a final volume of 400 µl T.E. buffer.

Before use, probes were boiled for 5 min, then cooled on ice for 5 min. Radiolabelled probe was added to a final volume of $1.8x10^6$ counts per minute ml^{-1} to either a hybridization tube or sealable plastic box and left to hybridize overnight. The following day, membranes were washed for 30 min in 5% SEN (40mM NaPi, 5% SDS, 1mM EDTA.), followed by washing 30 min in 1% SEN (40mM NaPi, 1% SDS, 1mM EDTA). If background radiation was high, the membrane was given a second wash with 1% SEN. Excess wash buffer was blotted on Whatman 3MM filter paper and the membranes placed on previously exposed X-ray film and sealed using Saran Wrap to keep the membrane moist (in case there was a need for a second hybridization) in a cassette containing either X-ray film or a phosphoimager screen (Amersham Biosciences). For phosphoimager screen analysis, cassettes were left at room temperature until developed (typically 5-6 hours initially), and then varied depending on the strength and clarity of the hybridization signal. For X-ray film analysis, cassettes were stored at –80°C until developed (typically the next day), then exposed for varying durations depending on the strength and clarity of the hybridization signal.

If membranes were to be re-probed, they were first stripped by removing them from the Saran Wrap, then placed in a Tupperware container and covered with boiling 0.1% SDS. The membrane was gently agitated in solution until it cooled to room temperature, then screened with a Geiger counter to determine if radioactive probe

remained. If background persisted, membranes were stripped a second time. If background still persisted, the membrane was discarded and a new DNA gel blot prepared. Stripped membranes were washed in 2x SSC before use, and then hybridized as above.

RNA Gel Blot Analysis

Total RNA extraction of plant leaf tissue was performed according to the manufacturer's instructions using the RNeasy Plant Mini Kit (Qiagen, Mississauga, ON). All glassware used was baked overnight at 400°C and all reagents were prepared using DEPC-treated water.

RNA was visualized on 1.2% formaldehyde agarose gels containing 50 ng μl^{-1} ethidium bromide. Prior to running samples, gels were equilibrated for 30 min in 1x Formaldehyde gel buffer. Prior to casting, all equipment was treated with 3% hydrogen peroxide.

For analysis of RNA transcripts, RNA gels were blotted onto Hybond-N+ membranes using 20x SSC. Membranes were cross-linked using a UV Stratalinker using the autocrosslink setting, then stored at 4°C in Saran Wrap. Prior to hybridization, membranes were wet with 2x SSC and prehybridized with 5 mL QuickHyb hybridization buffer (Stratagene, San Diego, CA) in a hybridization tube for 2 hours.

Transcripts were probed using the full length *WFCA-A1* cDNA. *WFCA-A1* cDNA was gel purified, then labeled using the rediprime™ II (Amersham Pharmacia) random prime labeling system with 5 μCi dCTP. Membranes were hybridized with the probe for a minimum of 24 hours. Afterwards, membranes were washed 4 times for 20

minutes with 0.2 SSC/0.1% SDS. Membranes were covered in Saran Wrap and exposed to a phosphoimager screen for various time lengths.

Protein Blotting

SDS-PAGE. SDS-PAGE (Sodium dodecyl sulphate Poly-Acrylamide Gel Electrophoresis) gels were prepared by adding 2.5 ml 1.5M Tris-HCI (pH 8.8), 100 µl 10% SDS, and 2.5 ml 30% Bis-Acrylamide together in a final volume of 10 ml. Solution was mixed and degassed for 15 minutes. Immediately before casting, 50 µl of fresh 10% APS (ammonium persulfate) and 5 µl of TEMED (N,N,N,N -Tetramethyl-Ethylenediamine) were added to the solution, mixed by inversion, and then pipetted into the casting stand. Casting was done in a BioRad Mini-Protean II Electrophoresis Cell (BioRad, Hercules, CA). The gel was covered with water-saturated isobutanol and left to polymerize for a minimum of 1 hour.

During this time, the 4% stacking gel was prepared by adding 2.5 ml 0.5M Tris-HCI (pH 6.8), 100 µl 10% SDS, and 1.33 ml 30% Bis-Acrylamide together in a final volume of 10 ml. The gel solution was mixed and degassed for 15 minutes. At this point, the isobutanol was poured off of the separating gel, then rinsed with distilled water and blotted with Whatman 3M filter paper to remove excess moisture. The plastic comb was placed in the casting cell. Immediately before casting the stacking gel, 50 µl of 10% APS 10 µl of TEMED were added to the gel solution and mixed by inversion. The gel solution was pipetted into the corner electrophoresis cell and the comb aligned. The stacking gel was left to polymerize for a minimum of 45 minutes before removing the comb.

The buffer chamber was assembled according to the manufacturer's instructions and running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS, pH 8.3) was added to the upper and lower buffer chambers. Air bubbles were removed and the gel wells were rinsed using running buffer. SDS-PAGE gels were pre-run for 30 minutes at 200 V. Samples were mixed with 2 volumes of loading buffer (62 mM Tris-HCl, pH6.8, 25% glycerol, 2% SDS, 0.0125% bromophenol blue, 5% β-mercaptoethanol [added just before use]) and heated at 95°C for 5 minutes. The samples were briefly centrifuged to collect the sample at the bottom of the tube, then added to gel wells using long-barreled pipette tips. Gels were run at 200 volts until the loading dye reached the bottom of the gel. Once electrophoresis was finished, the apparatus was disassembled and the gel prepared for electroblotting.

Electroblotting. Transfer buffer was prepared by combining 100ml 10x Transblot buffer (30.33g Tris base, 144g Glycine. Dilute to 1L with H2O. pH ~8.3), 200 ml MeOH, and 700 ml H2O, then chilled to 4°C. Gels were pretreated by soaking in transblot buffer for ~15 min. The Hybond-P PVDF membrane was pretreated by rinsing in 100% methanol (15 seconds), followed by rinsing in distilled water for 5 min, then soaked in transblot buffer for a minimum of 15 min.

The electroblotting apparatus was assembled by placing one transblot buffer-soaked foam square on the black plate, followed by 2-3 sheets of transblot buffer-soaked filter paper (cut to size). The gel was carefully placed face-down on top of the filter paper, which was then carefully covered by the membrane. The membrane was rolled with a clean glass rod to remove any air bubbles, then covered with 3 sheets of transblot

buffer-soaked filter paper, with air bubbles removed each time. Finally, a transblot buffer-soaked foam square was placed on top and the apparatus compressed together and secured. The apparatus was then placed into the BioRad Mini Trans-Blot Electrophoretic Transfer Cell system (BioRad, Hercules, CA) and filled with transblot buffer. The Trans-Blot was run at ~15 volts overnight with constant stirring at 4°C. The following day, the apparatus was disassembled and the membrane checked to ensure that the protein ladder had been successfully transferred to the membrane (with none visibly remaining in the gel). If transfer was incomplete, the Trans-Blot apparatus was reassembled and given a longer transfer time (at 100 volts for 2 h). Electroblotting was repeated until the marker was completely transferred to the membrane.

Anti-Serum Staining. The membrane was removed from the electroblotting apparatus and washed in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 5 minutes with gentle agitation. TBST was removed and the membrane was incubated with 20 ml 1% BSA-TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) for 90 min with gentle agitation. 20 µl of antibody serum (1 µl ml^{-1}) was added to the 1% BSA-TBST solution and incubated for 30 minutes with gentle agitation. The membrane was washed 3x with TBST for 10 min each, then reacted with 7 µl anti-IgG phosphatase (BioRad Blotting Grade Goat Anti-Rabbit-AP conjugate) for 30 min (3.5 µl per 10 ml TBST). The membrane was washed 3x with TBST for 10 min. Colour was developed by reacting the membrane with colour substrate (66 ml Nitroblue tetrozolium [50 mg ml^{-1} in 70% DMF], 10 ml AP [100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$], 33 ml 5-bromo-4-chloro-3-indolyl phosphate [50 mg ml^{-1} DMF]) for 15-25 min

in a foil-wrapped petri dish. Once membrane showed sufficient colour staining, it was removed, dried on Whatman 3M filter paper, and sealed in Saran Wrap.

Histochemical GUS Assay

Plants expected to express the *GUS*::*nptII* fusion gene (GUN) were stained to check for GUS expression. Healthy, green plant tissue (typically young rosette leaves) were harvested and immediately added to a 24-well plate containing ~200 µl of X-Gluc staining solution (1x Phosphate-buffered saline, 10 mM EDTA, 0.5 mM potassium-ferricyanide, 0.5 mM potassium-ferrocyanide, 1 mM X-gluc [5-bromo-4-chloro-3-indolyl-β-D-glucuronide], 1% Triton X-100). Tissue was incubated at 37°C for a minimum of 12 hours in order to allow sufficient time for the accumulation of indigo dye. Following incubation, X-Gluc solution was removed and replaced with a similar volume of 70% ethanol. Plates were again incubated at 37°C for several hours to destain and clarify the plant tissue. Typically tissues were given three separate ethanol treatments to fully clarify plant tissue. Plant tissue was visually scored based on the percentage of the surface area displaying the indigo dye.

DNA Sequencing

All sequencing of DNA, including plasmids and PCR amplicons, was handled by the DNA Services Unit at NRC PBI. Analysis was performed using the Lasergene DNAStar program suite (DNASTAR Inc., Madison, WI).

RESULTS

Restoration of the Positive FCA-γ Control

Initially, transformation of the *Arabidopsis fca-1* mutant with the *35S-FCA-γ* control developed at the John Innes Centre (Macknight et al., 2002) was desired for use as a positive control in this study. The *35S-FCA-γ* gene was graciously provided by Plant Bioscience Limited (P.B.L., Norwich, Norfolk, UK). However, there were two notable nucleotide substitutions in the coding sequence of the gene (Figure 6). These substitutions resulted in the replacement of alanine with threonine at position 266 and proline with leucine at position 296, both occurring in the second RNA Recognition Motif (RRM). As the RRMs are essential for proper functioning of the FCA peptide (Macknight et al., 1997), the replacement of a proline residue would likely have a significant impact on the tertiary structure of the RRM. Because of this, the effectiveness of the "positive" control was uncertain, so a restored version was produced.

To restore the correct sequence of the positive control, two primer sets (OL-5202/5203 and OL-5204/5205) were designed for use in site-directed mutagenesis. Plasmid pTH30-10 was used as the template. The 5202/5203 primer set corrects the ACA triplet (threonine) to the published GCA triplet (alanine). The 5204/5205 primer set corrects the CTT triplet (leucine) to the published CCT triplet (proline). Using these

primer sets, three mutagenic reactions were prepared: two single mutation reactions (one primer set each) and one double mutation reaction (both primer sets). Successful mutagenesis was confirmed by sequencing the mutagenized regions of the plasmids.

Both the original and corrected FCA-γ genes were cloned into the pHS737 binary vector for transformation into *fca-1* plants, in order to assess if the two point mutations affect the ability of the FCA-γ gene to complement the *fca-1* mutation. Unfortunately, due to a lack of time, I was unable to obtain these results.

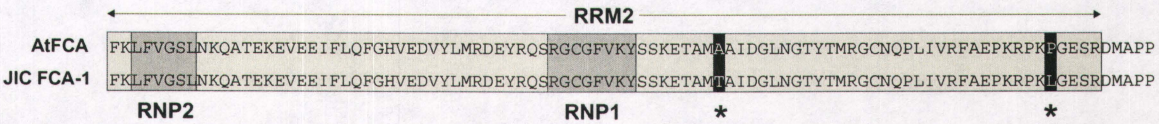

Figure 6. Comparison of the Published and Mutant *Arabidopsis FCA* Sequences. The positive control received from the John Innes Centre showed two major peptide polymorphisms. In order to correct these mutations, the gene was subjected to PCR-based site-directed mutagenesis. * = mutation, RNP1/2 = RNP Domain, RRM2 = 2nd RNA Recognition Motif.

Assembly of Plant Transformation Vectors

As *Triticum aestivum* is a hexaploid species, three genome-specific *WFCA* variants (one specific to the A, B, and D genomes) were prepared for complementation analysis in order to reduce the possibility that the inclusion of what may be a weaker or non-functional variant might lead to an erroneous conclusion regarding *WFCA* function.

Comparison of *FCA*-Like Genes from Various Plant Species. CLUSTALW alignment of FCA-γ-like peptide sequences from five species (*Arabidopsis thaliana* Z82989, *Brassica napus* AF414188, *Triticum aestivum* AY230398, *Oryza sativa* AY730687, and *Lolium perenne* AY654582) reveals a significant divergence between

monocot and dicot peptides, with approx. 40% identity conserved across the entire peptide for all five species (Table 3). *Arabidopsis* and *Brassica* share approximately 78% identity, and the three grass species share approximately 75% identity. However, alignments of the key FCA regions across all five species show a much higher conservation of amino acid residues, with the two RNA recognition motifs displaying 71-94% identity and the WW domain 81-100% identity. The putative C-terminal peptide appears to be non-existent in the *Brassica* ortholog, with only 5 amino acids conserved, yet the *Brassica FCA* gene has been shown to be processed correctly and complement the *Arabidopsis fca-4* mutant (Macknight et al., 2002). There have been no studies proving that the C-terminal peptide is required for FCA function, and it is interesting to note that the *Brassica FCA* ortholog appears functional despite its apparent lack of the C-terminal peptide. Whether or not the C-terminal peptide plays a critical role in FCA function remains to be seen. Comparison of the C-terminal peptide across the remaining four species (*Arabidopsis, Triticum*, *Oryza*, and *Lolium*) shows conservation of 47-97% identity. Taken together, these alignments indicate that the putative FCA peptides in the grass species show a significant amount of divergence in the overall protein, but share a significant amount of conservation with specific regions of the *Arabidopsis* FCA protein (RRM1 and 2, WW domain, and CTP) and a high degree of similarity to each other. Based on the considerable similarity between WFCA and FCA, as well as similar proteins from other plant species, there is a good possibility that *WFCA* is a wheat ortholog of *FCA*, or serves a similar function.

Table 3. Conservation of FCA Peptide Identity Across Five Species.

Comparison (vs.)		Overall	RRM1&2	WW	CTP
Size		~747 aa	181 aa	26 aa	19 aa
Arabidopsis	*Brassica*	78 %	94 %	96 %	*28 %
	Triticum	38.5 %	76 %	85 %	50 %
	Oryza	40 %	75 %	81 %	58 %
	Lolium	40 %	72 %	85 %	47 %
Brassica	*Triticum*	40 %	75 %	85 %	*28 %
	Oryza	39 %	74 %	81 %	*28 %
	Lolium	39 %	71 %	85 %	*22 %
Triticum	*Oryza*	78 %	90 %	96 %	90 %
	Lolium	84 %	90 %	100 %	95 %
Oryza	*Lolium*	75 %	83 %	96 %	84 %

	Arabidopsis	*Brassica*	*Triticum*	*Oryza*	*Lolium*
Peptide size	747 aa	715 aa	741 aa	738 aa	668 aa

Peptide sequence identity was analyzed using the Lasergene software suite and compared across a representative gamma-form member of five different species. While overall sequence identity varies over the whole peptide, there is a strong conservation of sequence in the key regions with a notable exception of the C-terminal peptide in *Brassica*.
RRM = RNA Recognition Motif, WW = WW Domain, CTP = C-terminal peptide.

Construction of a D-type Hybrid *WFCA*. Unfortunately, there was no full-length D-type cDNA available for use in this study. The longest D-type cDNA sequence was the cloned RT-PCR product 1413-A1, containing D-type sequence spanning the last 88 nt of exon 1 to the end of exon 17 (M. Gao, T. Hoffman, A.E. Limin, D.B. Fowler, and G. Selvaraj, unpublished results). In order to assess if the D-genome *WFCA* was able to complement *fca-1*, a synthetic *WFCA* gene was constructed to mimic the D-type *WFCA* (Figure 7). Plasmids pMG-T7 (full-length A-type *WFCA*) and pMG-1412-A1 (partial D-type *WFCA*) were digested with HindIII for 1 hour, then heat-killed by incubating the reaction tubes at 75°C for 15 min. The HindIII endonuclease was used instead of MfeI as the D-type *WFCA* lacks the MfeI site present in the A- and B-type *WFCA*. The HindIII

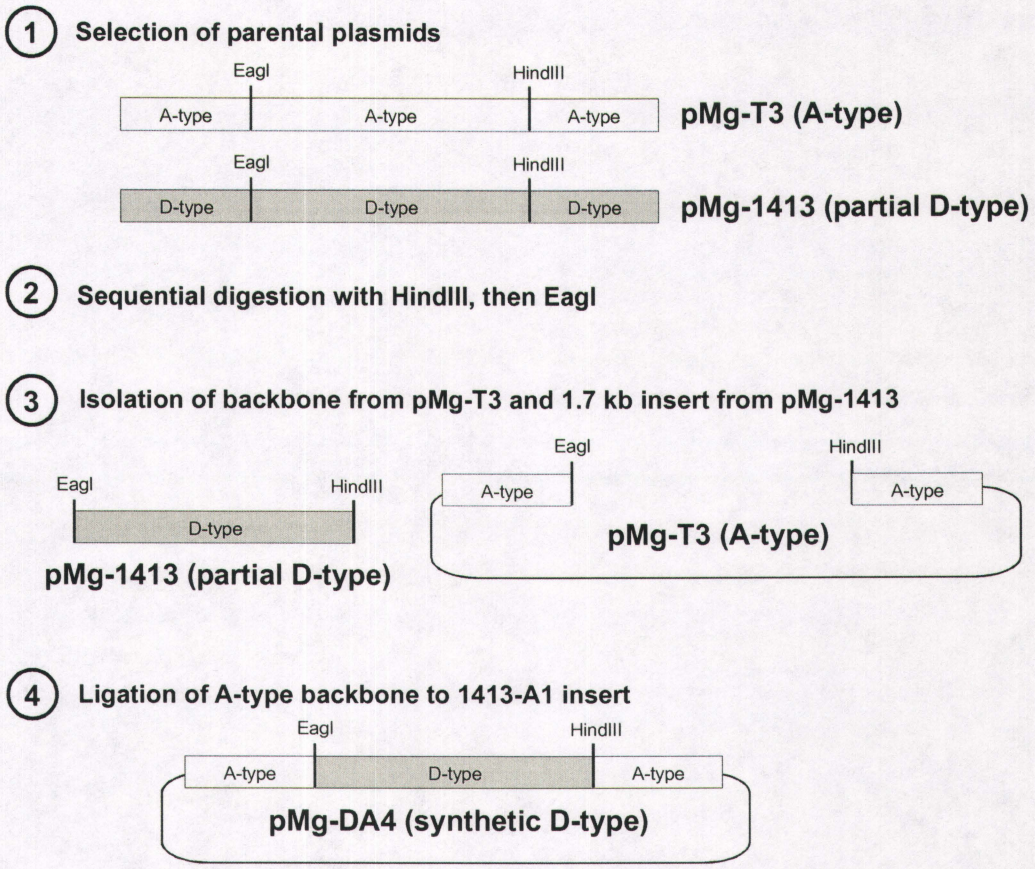

Figure 7. Digestion Strategy for Construction of the Hybrid ADA *WFCA*.
As there was no full-length D-type *WFCA* sequence available for complementation, the partial cDNA pMg-1413 containing most of the D-type *WFCA* sequence was digested with EagI and HindIII, and the D-type sequence was subcloned into the A-type *WFCA-A1* to produce an ADA-hybrid.

site occurs in Exon 15, after the fourth and fifth TR regions. Digestion reactions were ethanol precipitated by and the resuspended DNA was then digested with EagI for 1 hour. The 1.7 kb D-type *WFCA* insert and 5.5 kb *WFCA-A1* backbone were gel purified, then ligated together to produce the synthetic D-type *WFCA* gene. The resulting plasmid (pMg-DA4) was screened and sent for sequencing to confirm that the replacement was correct.

The synthetic D-type *WFCA* gene pMg-DA4 contains the *WFCA*-D-type sequence from exons 2 to 14, along with the terminal 65 nt of exon 1 and 82 nt of exon

15. The synthetic D-type *WFCA* contains A-type TR1 and TR2 and D-type TR3, TR4, TR5, and D-type RNA Recognition Motifs and WW Domain.

Construction of Binary Vectors. *Arabidopsis fca-1* mutants were transformed with three *WFCA* variants, representing each of the three hexaploid genomes (A, B, and D), the *AtFCA* control, and the control vector (pHS737). The A-type *WFCA* selected was *WFCA-A1* (Accession #AY230398). The B-type *WFCA* selected was *WFCA-B2* (Accession #AY230400) The D-type *WFCA* is a synthetic *WFCA* gene constructed as described above. The binary vector selected for use is pHS737, which contains a double-35S promoter and the polyA terminator (Figure 8). The double-35S promoter was chosen to ensure a high level of *WFCA* expression, both for analysis of its effect on flowering time and to allow for detection of potential pleiotropic effects of *WFCA* expression. In addition, the pHS737 plasmid contains a *GUS::nptII* fusion gene (*GUN*) under the control of the double-35S promoter. The *GUN* fusion results in transformed plants possessing resistance to kanamycin due to the detoxification by *nptII* as well as producing an indigo dye when *GUS* reacts with a suitable substrate (Jefferson, 1987). Since both *GUN* and the *WFCA/FCA* gene are located on the T-DNA region that is inserted into transformed plants, plants expressing the *FCA*-like genes should likewise contain the *GUN* marker.

Three different genome-associated *WFCA* types were initially selected in order to ensure that genome-specific differences including tri-nucleotide repeat (TR) variation did not lead to an erroneous conclusion. *Vrn-A1* appears to have the strongest influence on the vernalization requirement in wheat, and there may be similar dominant relationships among the interacting factors (Toth et al., 2003). Variation in tri-nucleotide

repeats has been shown to have specific consequences in mammalian systems including the severity of myotonic dystrophy (Ashley and Warren, 1995). If similar variations compromise the action of certain *WFCA* genes, it may be erroneously assumed to have no effect in the *Arabidopsis* system due to an initial oversight.

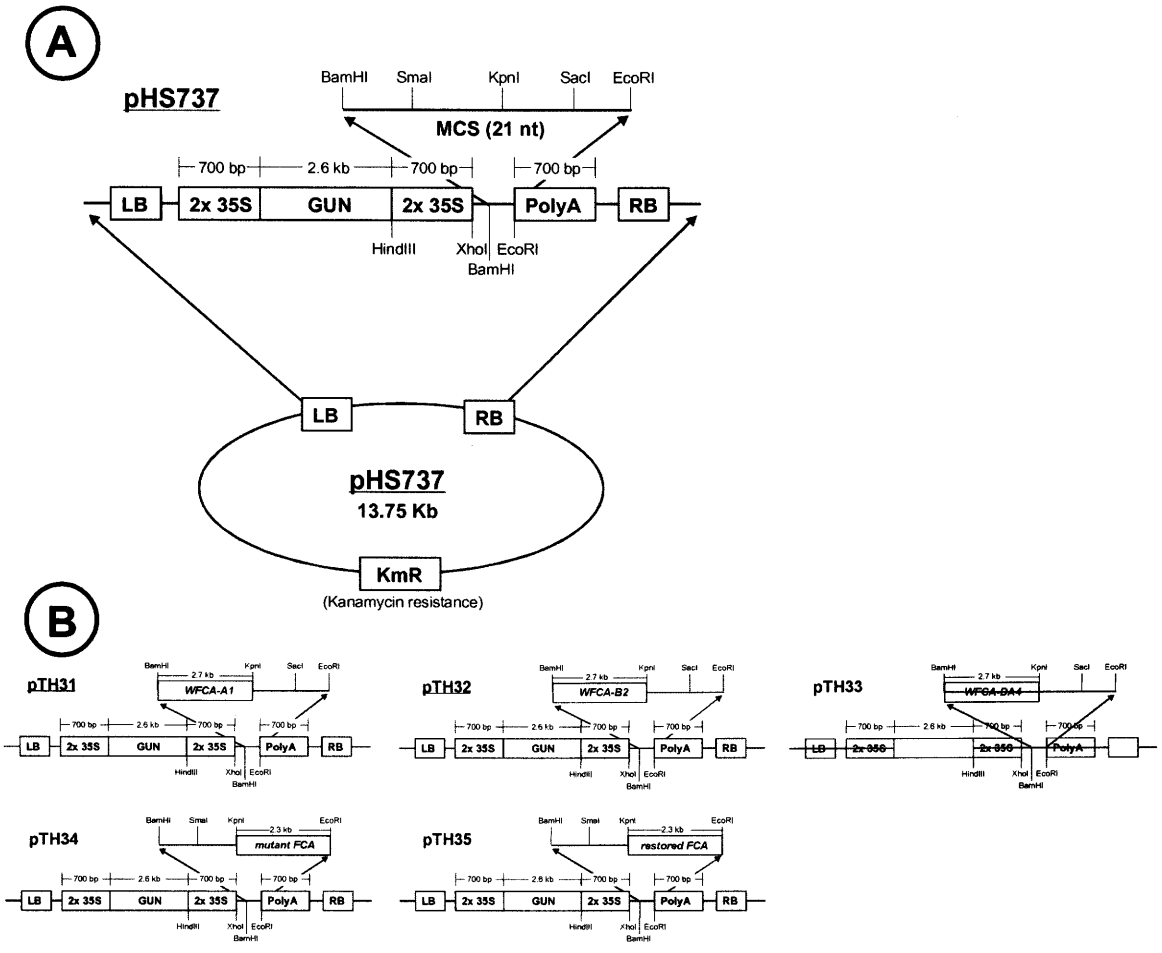

Figure 8. Plasmid Maps for Binary Vectors.
Note: maps are not drawn to scale.
A) The binary vector pHS737 is a 13.75 kb plasmid vector containing the left and right border sequences from *Agrobacterium tumefaciens* and the GUN Kanamycin-resistance selection marker. All genes for transformation were subcloned into the span between the BamHI and KpnI restriction sites of the Multiple Cloning Site (MCS).
B) The inserts for each transformation vector (pTH31-35) are shown cloned into the pHS737 MCS, and will be expressed via the action of the double-35S promoter.

In total, 6 binary vectors were prepared for transformation into *Arabidopsis fca-1* mutants (Figure 8). Three *WFCA* variants (A, B, and D), designated as pTH31, 32, and 33, were produced, but due to time limitations only pTH31 was evaluated for its effect on flowering behaviour. Since the *FCA-γ* control gene received possessed notable mutations in its sequence, two vectors were developed containing the unmodified gene as received (pTH34) and a corrected *FCA-γ* control based on the published *FCA-γ* sequence (pTH35) (Figure 8). The pHS737 vector was used as the vector control.

Confirmation of the *fca-1* Mutation.

To ascertain that the *Arabidopsis* plants transformed in this study contained the published *fca-1* mutation, rosette leaves were collected from plants, immersed in liquid nitrogen, ground to a fine powder, and stored at –80°C. Later, genomic DNA was extracted using the CTAB-buffer method. Primer pair OL-5260 and OL-5261 (DNA Services Unit, PBI NRC) was designed to amplify the region of *FCA* containing the point mutation. PCR required the use of a low KCl PCR buffer (optimum pH 8.3, 1.5 mM $MgCl_2$, 25 mM KCl) and was performed using the standard temperature profile.

Sequences were aligned using the MegAlign program (DNASTAR Inc., Madison, WI) and compared with the genomic *FCA* sequence obtained from EMBL (Accession Z82992.1, EMBL CAB05391). All *fca-1* plants screened had the C-T substitution at nucleotide 1405, while the Landsberg erecta controls (Ler-0 genotype) did not. This substitution produces a stop codon at residue position 468, producing a non-functional FCA peptide containing both RRMs but missing the WW Domain and C-terminal peptide (Macknight et al., 1997).

Flowering Analysis of Transgenic Plants

Flowering Comparison. The flowering criterion for this study was the time taken from sowing until the opening of the first flower (as per Koornneef et al., 1991). After flowering, the final rosette leaf numbers were recorded (as per Macknight et al., 1997). In order to assess the effects of *WFCA-A* in *Arabidopsis fca-1* plants, a T1 generation needed to be produced.

Due to time constraints, in this study only putative *WFCA-A* T1 transgenic plants were analyzed for their flowering behaviour. In total, 424 putative T1 transgenics, 37 Ler-0 controls, and 36 *fca-1* controls were grown to maturity. Soil composition affected the morphology of the plant, with plants grown in Sunshine Mix #3 typically displaying a smaller overall stature when comparison to plants grown in Rediearth. Additionally, plants grown in Sunshine Mix #3 displayed a noticeable purpling of the rosette leaves when compared to the Rediearth-grown plants, which typically displayed the expected green colour, although some purple-colour was seen in some Rediearth-grown T1 plants as they began to senesce. However, Sunshine Mix #3-grown *Arabidopsis* plants were noticeably smaller than those grown in Rediearth, so the dramatic difference in colour may be complicated by the reduced size of their rosette leaves, which would be expected to show a darker colour if the pigments were concentrated in a smaller area.

Regardless of the choice of soil media, the early-flowering Ler-0 controls bolted with an average FLN of 8.7, late-flowering *fca-1* controls had an average FLN of 14.8, and the overall FLN for the *WFCA-A* T1 progeny was 14.3 (Table 4, Figure 9). Of the 424 *WFCA-A* T1 plants analyzed, only one displayed a FLN less than 11. Analysis of variance for the flowering data indicated that there were significant differences between

all three backgrounds (P<0.001), indicating that although the *WFCA-A* transgenic population is not homogenous, it does have a promotive effect on the floral transition in *Arabidopsis fca-1* mutants. However, *WFCA-A* appears insufficient to fully restore flowering time in comparison to the Ler-0 control as only a single plant displayed a FLN consistent with the mean FLN for Ler-0. ANOVA between the T1 population FLN and the *fca-1* control population FLN also showed strong significance (P<0.05). Pooled standard deviation for both ANOVA tests was 1.3.

Table 4. Analysis of T1 Progeny and Flowering Controls.

Background	Soil	# Plants	Min FLN	Max FLN	Avg FLN	Avg DtB[a]
Ler-0 control	Rediearth	18	7	10	8.8 ± 0.8	22.3 ± 3.6
	Sunshine	19	7	10	8.5 ± 0.9	21.8 ± 0.8
	All	37	7	10	8.7 ± 0.8	22.1 ± 2.6
fca-1 control	Rediearth	18	13	17	15.0 ± 1.1	26.5 ± 1.5
	Sunshine	18	12	16	14.6 ± 1.2	27.4 ± 1.8
	All	36	12	17	14.8 ± 1.2	27.0 ± 1.7
Wfca-A T1	Rediearth	227	11	18	14.5 ± 1.4	26.8 ± 1.8
	Sunshine	197	9	17	14.2 ± 1.3	27.4 ± 2.6
	All	424	9	18	14.3 ± 1.3	27.1 ± 1.6

Total leaves (including cotyledons) were counted for all T1 progeny once the plant had bolted. Unfortunately, the *fca-1* controls did not display the high FLN expected, despite confirmation of the *fca-1* mutation in the parental plant.
a) DtB = Days to Bolting

In both soil media types, the average rosette FLN and number of days to flowering were similar, although the deviation in the flowering date did show some impact of the media. In general, some of the plants grown in Sunshine Mix #3 took longer to germinate than those grown in Rediearth. However, the similarity of the FLN in both media types indicates that the soil mixture had little effect on the level of developmental growth attained by the plant during its juvenile stage.

Results of GUS Staining. The *GUN* cassette expresses the glucuronidase (GUS) enzyme, which produces an indigo dye when it acts on a solution containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc). Leaves were harvested from bolting plants and immersed in X-Gluc solution in sealed 24-well plates and left to stain for a minimum of 12h. All of the Ler-0 and *fca-1* controls (which were not expected to express GUS) did not produce the blue precipitate.

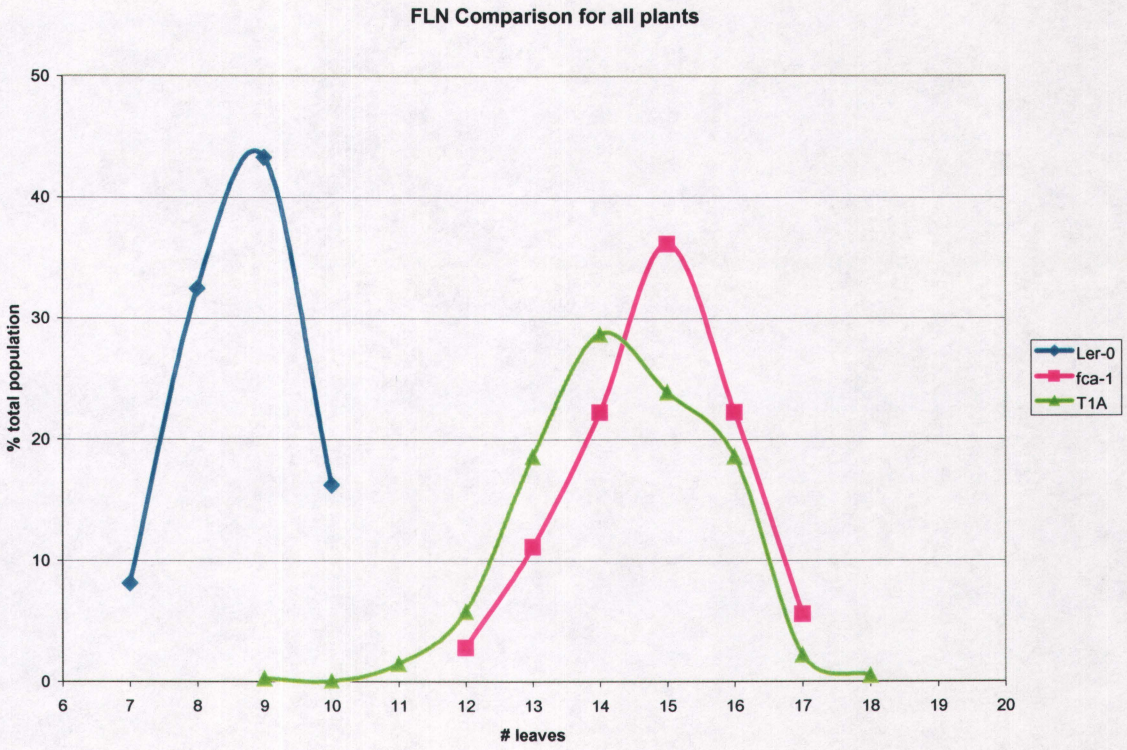

Figure 9. Comparison of FLN for *WFCA-A* T1 Plants.
FLN was recorded for plants of each genetic background (Ler-0, *fca-1*, and T1 progeny from *WFCA-A* transformed *fca-1* lines) and adjusted for the total percentage of the population. There was a slight but statistically significant shift in the mean between the unselected T1 population and the *fca-1* control, as determined by one-way ANOVA (Standard Deviation was 1.3). Only 1 plant from the T1 population had a FLN (9) similar to the FLN of the Ler-0 control.

GUS staining of T1 progeny indicated that there was a range of GUS expression in transgenic *fca-1* mutants (Figure 10). An analysis of 47 putative transgenic plants had

4 negative (no stain), 22 with weak staining (25% of leaf area or less), and 21 with strong staining (more than 25% of leaf area). If there was one *WFCA-A* transgene inserted into the *fca-1* parent, an expected segregation ratio should be approximately 3 GUS positive plants to 1 GUS negative plant. Instead, the ratio was approximately 10:1, indicating the possibility of multiple *WFCA-A* insertions. To obtain an accurate number, DNA gel blot analysis will be needed. Regardless of the copy number, I was able to detect and confirm the presence of the *GUN* marker in the T1 population, which indicates that these plants also contain the *WFCA-A* transgene.

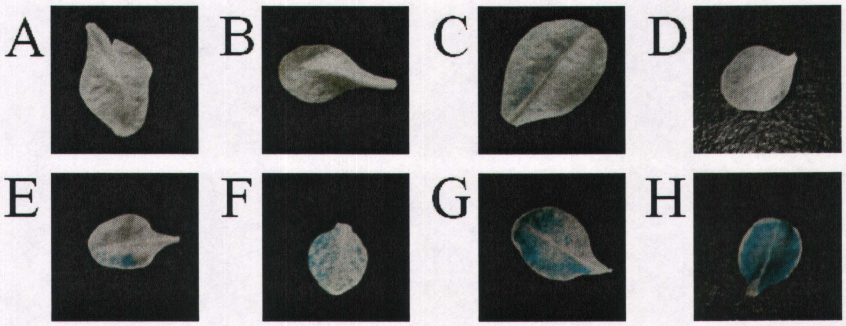

Figure 10. Comparison of GUS Staining in *WFCA-A* T1 Plants.
GUS staining resulted in a range of staining intensities, which had no correlation with the FLN or days to bolting displayed by the T1 progeny. Staining was rated on a scale from 0-4, with 0 (GUS -) indicating no staining while 4 (GUS++++) indicated a strong, nearly complete staining of the leaf.
A) Ler-0 control [GUS -]. B) *fca-1* control [GUS -].
C,D) Light GUS staining [GUS +]. E,F) Moderate GUS staining [GUS ++].
G) Major GUS Staining [GUS+++]. H) Strong GUS staining [GUS ++++].

DISCUSSION

Analysis of soil grown T1 progeny from *35S::WFCA-A* transformed *fca-1* mutants suggests that *WFCA-A* expression has a small effect on the flowering time when transformed into *Arabidopsis fca-1* mutants. While only one plant out of 424 displayed a FLN less than 11, the unselected population bolted on average with ½ rosette leaf less than the *fca-1* controls. It is important to note that this study did not ascertain if the B- and D-type *WFCA* variants were able to complement the *fca-1* mutation, and if so, if there were different strengths of complementation relating to their structural differences from the *WFCA-A* gene. However, based on the results of *WFCA-A* expression in *Arabidopsis fca-1* mutants, it is possible that WFCA-A may be involved in a different capacity in wheat, or interact with different proteins to accelerate flowering. If these proteins are absent in the *Arabidopsis* system, or have sufficiently diverged from the *Arabidopsis* system to the point where the WFCA protein is unable to interact with dicot floral integrators, it would explain why *WFCA-A* was unable to strongly complement the *fca-1* mutation.

Loukoianov et al. (2005) demonstrated the existence of an alternative system for vernalization response in wheat, where in contrast to the *Arabidopsis* model (Figure 1), the vernalization-repressed Zinc finger-CCT *VRN-2* gene acts to repress the expression of the *AP1*-like *VRN-1* gene, which promotes the flowering transition in wheat (Loukoianov et al., 2005; Yan et al., 2003). In light of this divergence from the

prevailing *Arabidopsis* model, the existence of this separate system for vernalization response raises questions as to the extent of conservation between floral pathway integrators among monocot and dicot species. If the vernalization response has sufficiently diverged in grass species, it is quite possible that the functions of some of the autonomous pathway genes may also have diverged, and may therefore possess altered or severely reduced functionality in the other system.

A recent study indicates that *FCA*-like genes isolated from grass species may have divergent functions. Lee et al. (2005) have demonstrated that a single-copy *FCA*-like gene isolated from rice, designated *OsFCA*, was only able to partially rescue the *fca-1* mutation. In that study, a full-length *OsFCA* cDNA was transformed into *Arabidopsis fca-1* mutants, which resulted in the transgenic plants in the T0 generation displaying a range of phenotypes, with 14 of the 50 T0 plants displaying severely curled leaves and dying before setting seed and the remainder "evidenced phenotypic rescue to different degrees, ranging from almost complete rescue, to no rescue whatsoever" (Lee et al., 2005). Examination of selected T1 progeny showed FLNs of 16.9-20.9, the *fca-1* control 26.5 $\pm$ 4.0, and the Ler-0 control 9.6 $\pm$ 1.1. Taken together, the transgenic plants expressing OsFCA had an FLN reduction of approximately 5 to 9 leaves. In addition, the study showed that while *OsFCA* showed the characteristic autoregulatory loop (where the FCA protein regulates its own mRNA levels by promoting alternative splicing, resulting in the non-functional β-form transcript) in *Oryza sativa*, it was unable

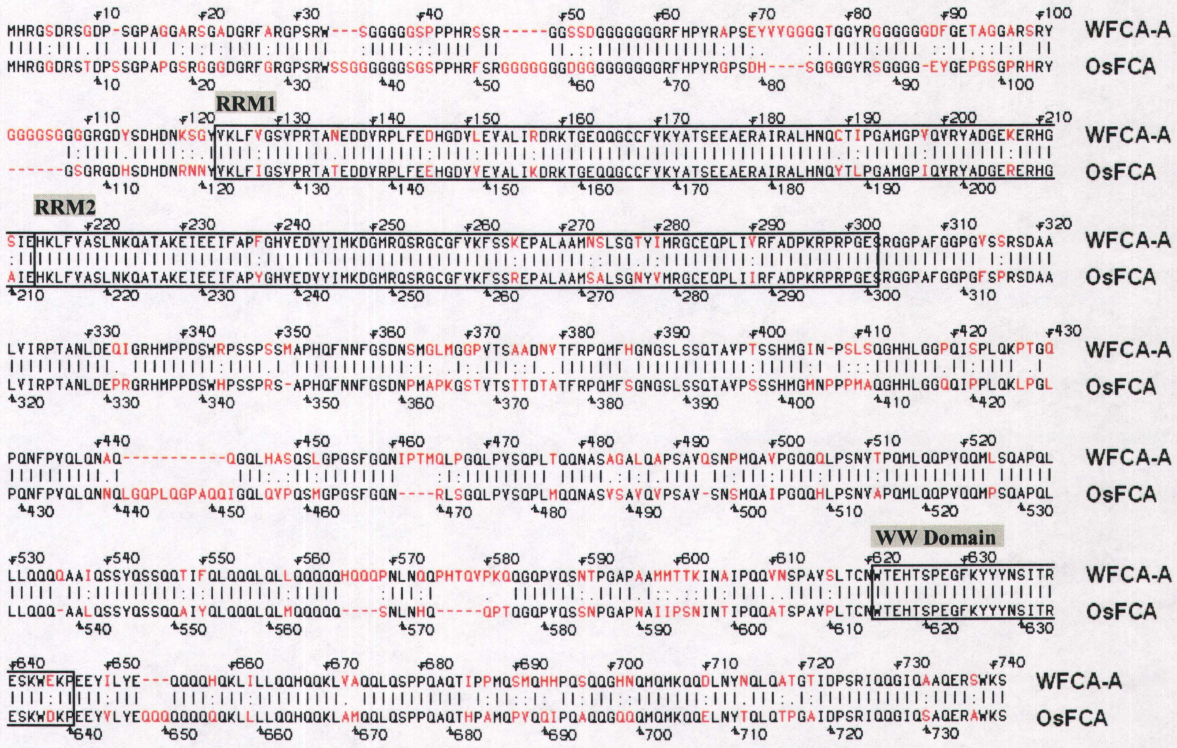

Figure 11. Comparison of WFCA and OsFCA Peptide Sequences.
The OsFCA peptide was able to partially complement the *Arabidopsis fca-1* mutation (Lee et al., 2005). As shown here, there are several differences between the two peptide, but the RNA Recognition Motifs and the WW domain are quite similar. Interestingly, the N-terminal end of the peptides shows a fair amount of divergence, with the OsFCA having a pronounced Glycine-rich region.

to mediate *FCA-γ* expression (which would produce a high level of the short *FCA-β* transcript) in *Arabidopsis*. However, analysis of transcript levels for *SOC1* and *FLC* indicated that while OsFCA upregulated *SOC1* levels, it was insufficient to repress *FLC*. Taken together, these experiments indicate that *OsFCA* is unable to reduce *FLC* expression and regulate *FCA* expression, both key parts of *FCA* function. Previously, upregulation of *SOC1* during *FCA* expression was assumed to be the result of its effects on *FLC*, but the results of this study may indicate that *FCA*-like genes can induce other flowering-related genes (Quesada et al., 2005; Lee et al., 2005). Lee et al. (2005) speculated that the lack of strong complementation was due to OsFCA being unable to

interact with the *Arabidopsis* FY, or that it may function in other capacities in rice, such as the regulation of environmental signals.

The peptide sequence of WFCA shows a higher degree of similarity to OsFCA than *Arabidopsis* FCA, suggesting that WFCA and OsFCA may perform a similar function to each other as opposed to *FCA* (Figure 10). The 5'region of FCA-like proteins shows a considerable amount of diversity among species, which may impact its ability to complement dicot *fca* mutations. Alternately, variation in the RRM or WW domains may prevent monocot-type FCA-like proteins from interacting with the *Arabidopsis* FY. These regions are essential for the proper function of FCA, and are not completely conserved across species. Monocot-type *FCA*-like genes may interact with different target proteins or phytohormones, including ABA, or recognize different targets. No experiments (such as the Far Western analysis described by Simpson et al., 2003) have been performed to test the compatibility between the WW domain of OsFCA and *Arabidopsis* FY. If monocot *FCA*-like genes are unable to interact with FY, OsFCA would be functionally unable to reduce the expression of *FLC* or participate in the FCA autoregulatory loop (Lee et al., 2005). This would support the conclusion that WFCA may exhibit a divergent function in the monocot system. The ability of *OsFCA* to promote *SOC1* expression may be either a separate function of FCA and FCA-like proteins, or it may be that OsFCA has different targets and/or responses than FCA.

CONCLUSIONS

Based on the results of the T1 progeny analysis, it appears that *WFCA-A* expression in *Arabidopsis fca-1* mutants produces a small yet significant acceleration of the flowering transition. This indicates that WFCA likely has a partial retention of the flowering promotion action of FCA, but based on this study we cannot conclude whether or not it is related to its action in conjunction with FY. More work is needed before *WFCA* genes can be assumed to fulfill a similar role to FCA in the floral transition in *Triticum aestivum*. These results suggest that it is involved to some degree in flowering promotion. Although the results seen in the WFCA-A T1 population, where the FLN decreased by approximately half a leaf over the transgenic and non-transgenic progeny, may seem to be a slight difference, this may simply be a result of the *WFCA* transgene being unable to fully function within the *Arabidopsis* system, as was suggested in the study of OsFCA (Lee et al., 2005). In that study, it was assumed that the non-native monocot-based *FCA* was unable to fully interact with the necessary complexes required to exhibit full complementation similar to that seen with the Brassica *FCA* ortholog (Macknight et al., 2002).

Unfortunately, in this study the late-flowering *fca-1* negative control had a lower FLN than was expected, so additional experimental replicates will be necessary to draw a firm conclusion as to whether or not *WFCA-A* expressing T1 progeny show a significant shift in FLN. The reason for the reduced FLN of the *fca-1* controls may be

due in part to the size of the individual cells of the trays used to grow the plants (see Appendix 1, T1 Media-Transferred Progeny). In addition, there is still the question of whether the B-type or D-type *WFCA* genes show differing levels of promotive behaviour. Along these lines, it is also important to determine if an ortholog for FY exists in a monocot system (the only match is a predicted protein in *Lolium perenne*, Accession #AY654583 (Genbank, http://www.ncbi.nlm.nih.gov/Genbank/)), and if monocot *FCA*-like genes (including *WFCA*) are able to interact with the *Arabidopsis* FY. Currently, the question of what proteins and/or metabolites with which *WFCA* is able to interact appears to be of critical importance in determining the role of *WFCA* in the wheat system. From the recent work of Razem et al. (2004, 2006) it is likely that ABA plays a role in the function and/or regulation of *FCA*-like genes, as well as in the flowering process. Although WFCA has not been shown to interact with ABA, due to the strong homology of WFCA with ABAP1 (indeed, ABAP1 and WFCA may be homologs) it is extremely likely that WFCA possesses the same capacity to bind ABA (Razem et al., 2006). We still possess imperfect knowledge of the flowering process and its regulators, and ultimately it seems that an elaborate study is needed to analyze the effects of monocot *FCA*-like genes in a model monocot system, rather than relying the use of the better-understood model dicot *Arabidopsis*. By developing a usable monocot model system, it would ensure that all proteins and metabolites necessary to produce a flowering response are present in the model system, reducing the chances of false conclusions based on differences due to the divergence that occurred between the two systems.

In light of the growing differences in the behaviour of flowering-related genes between monocots and dicots, to perform robust and reproducible analyses, the selection

of a model monocot system seems necessary (Yano et al., 2000; Lee et al., 2005). The intertwined processes of the floral transition and flowering have been demonstrably shown to be dynamic, complex, and possess significant amounts of redundancy and duplication (Hay and Ellis, 1998; Jack, 2004; Komeda, 2004; Levy and Dean, 1998). In light of this complexity, performing gene-by-gene analysis in the dicot system seems an insufficient methodology for any large-scale study of monocot flowering behaviour. While genes with highly similar functions will demonstrate obvious, positive results, we will ultimately need to understand and model the differences between flowering behaviours exhibited by monocot and dicot plants. There is still a significant lack of understanding surrounding the regulation of the flowering transition within a monocot system, which strongly limits our ability to work at improving several economically and nutritionally important crop species, as well as manipulating other monocot and cereal plants for use in crop development.

REFERENCES

Ashley, C.T., and Warren, S.T. (1995) Trinucleotide repeat expansion and human disease. *Annual Review of Genetics* **29**, 703-728.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A., and Dean, C. (2004) Vernalization requires epigenetic silencing of *FLC* by histone methylation. *Nature* **427**, 164-167.

Blackwell, J.R., and Horgan, R. (1991) A novel strategy for production of a highly expressed recombinant protein in an active form. *Federation of European Biochemical Societies Letters* **295**, 10-12.

Blazquez, M., Koornneef, M., and Putterill, J. (2001) Flowering on time: genes that regulate the floral transition. *European Molecular Biology Organization Reporter* **2**, 1078-1082.

Caldwell, K.S., Dvorak, J., Lagudah, E.S., Akhunov, E., Luo, M.C., Wolters, P., and Powell, W. (2004) Sequence polymorphism in polyploid wheat and their D-genome diploid ancestor. *Genetics* **167**, 941-947.

Chen, L., Cheng, J.C., Castle, L., and Sung, Z.R. (1997). *EMF* genes regulate Arabidopsis inflorescence development. *Plant Cell* **9**, 2011–2024.

Chouard, P. (1960) Vernalization and its relations to dormancy. *Annual Review of Plant Biology* **11**, 191-238.

Clough, S.J., and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735-743.

Danyluk, J., Kane, N.A., Breton, G., Limin, A.E., Fowler, D.B. and Sarhan, F. (2003) TaVRT-1, a putative transcription factor associated with vegetative to reproductive transition in cereals. *Plant Physiology* **132**, 1849-1860.

Dubcovsky, J., Lijavetzky, D., Appendino, L., and Tranquilli, G. (1998) Comparative RFLP mapping of *Triticum monococcum* genes controlling vernalization requirement. *Theoretical and Applied Genetics* **97**, 968-975.

Dvorak, J. (1998). Genome analysis in the *Triticum Aegilops* alliance. In: *Proceedings of 9th International Wheat Genetics Symposium, University of Saskatchewan, Saskatoon* **1**, 8-11.

Finnegan, E.J., Kovac, K.A. Jaligot, E. Sheldon, C.C., Peacock, W.J., and Dennis, E.S. (2005) The down-regulation of *FLOWERING LOCUS C (FLC)* in plants with low levels of DNA methylation and in vernalized plants occurs by distinct mechanisms. *Plant Journal* **44**, 420-432.

Finnegan, E.J., Genger, R.K., Kovac, K., Peacock, W.J. and Dennis, E.S. (1998) DNA methylation and the promotion of flowering by vernalization. *Proceedings of the National Academy of Sciences USA* **95**, 5824-5829.

Good, P. (1995) A conserved family of *ELAV*-like genes in vertebrates. *Proceedings of the National Academy of Sciences USA* **92**, 4557-4561.

Gu, Y.Q., Coleman-Derr, D., Kong, X., and Anderson, O.D. (2004) Rapid genome evolution revealed by comparative sequence analysis of orthologous regions from four *Triticeae* genomes. *Plant Physiology* **135**, 459-470.

Hay, R.K.M. and Ellis, R.P. (1998) The control of flowering in wheat and barley: what recent advances in molecular genetics can reveal. *Annals of Botany* **82**, 541-554.

Heyne, E.G. (editor) (1987) Wheat and Wheat Improvement, Second Edition. American Society of Agronomy, Madison, WI.

Huang, S., Sirikhachornkit, A., Su, X., Faris, J., Gill, B., Haselkorn, R. and Gornicki, P. (2002) Genes encoding plastid acetyl-CoA carboxylase and 3-phosphoglycerate kinase of the *Triticum/Aegilops* complex and the evolutionary history of polyploid wheat. *Proceedings of the National Academy of Sciences USA* **99**, 8133-8138.

Isidore, E., Scherrer, B., Chalhoub, B., Feuillet, C. and Keller, B. (2005) Ancient haplotypes resulting from extensive molecular rearrangements in the wheat A genome have been maintained in species of three different ploidy levels. *Genome Research* **15**, 526-536.

Jack, T. (2004) Molecular and genetic mechanisms of floral control. *Plant Cell* **16**, S1-S17.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter* **5**, 387-405.

Kato, K., Miura, H., and Sawada, S. (1999) QTL mapping of genes controlling ear emergence time and plant height on chromosome 5A of wheat. *Theoretical and Applied Genetics* **98**, 472-477.

Komeda, Y. (2004) Genetic regulation of time to flower in *Arabidopsis thaliana*. *Annual Review of Plant Biology* **55**, 521-535.

Koncz, C., and Schell, J. (1986). The promoter of TL-DNA gene 5 controls the tissue-specific expression of chimaeric genes carried by a novel type of *Agrobacterium* binary vector. *Molecular and General Genetics* **204**, 383-396.

Koornneef, M., Hanhart, C.J., and van der Veen, J.H. (1991) A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. *Molecular and General Genetics* **229**, 57-66.

Lee, J.H., Cho, Y.S., Yoon, H.S., Suh, M.C., Moon, J., Lee, I., Weigel, D., Yun, C.H., and Kim, J.K. (2005) Conservation and divergence of *FCA* function between *Arabidopsis* and rice. *Plant Molecular Biology* **58**, 823-838.

Levy, Y.Y., and Dean, C. (1998) The transition to flowering. *Plant Cell* **10**, 1973-1990.

Li, W., Zhang, P., Fellers, J.P., Friebe, B., and Gill, B.S. (2004) Sequence composition, organization, and evolution of the core *Triticeae* genome. *The Plant Journal* **40**, 500-511.

Limin, A.E., and Fowler, D.B. (2002) Developmental traits affecting low-temperature tolerance response in near-isogenic lines for the vernalization locus *Vrn-A1* in wheat (*Triticum aestivum* L. em Thell). *Annals of Botany* **89**, 579-585.

Lin, K.T., Lu, R.M. and Tarn, W.Y. (2004) The WW Domain-containing proteins interact with the early spliceosome and participate in pre-mRNA splicing *in vivo*. *Molecular and Cellular Biology* **24**, 9176-9185.

Lin, S.I., Wang, J.G., Poon, S.Y., Su, C.L., Wang, S.S. and Chiou, T.J. (2005) Differential regulation of *FLOWERING LOCUS C* expression by vernalization in cabbage and *Arabidopsis*. *Plant Physiology* **137**, 1037-1048.

Lisbin, M.J., Gordon, M., Yannoni, Y.M. and White, K. (2000) Function of RRM domains of *Drosophila melanogaster* ELAV: RNP1 mutations and RRM Domain replacements with ELAV family proteins and SXL. *Genetics* **155**, 1789-1798.

Loukoianov, A., Yan, L., Blechl, A., Sanchez, A., and Dubcovsky, J. (2005) Regulation of *VRN-1* vernalization genes in normal and transgenic polyploid wheat. *Plant Physiology* **138**, 2364-73.

Macknight, R., Bancroft, I., Page, T., Lister, C., Schmidt, R., Love, K., Westphal, L., Murphy, G., Sherson, S., Cobbett, C., and Dean, C. (1997) FCA, a gene controlling flowering time in *Arabidopsis*, encodes a protein containing RNA-binding domains. *Cell* **89**, 737-45.

Macknight, R., Duroux, M., Laurie, R., Dijkwel, P., Simpson, G.G. and Dean, C. (2002) Functional significance of the alternative transcript processing of the *Arabidopsis* floral promoter *FCA*. *Plant Cell* **14**, 877-888.

Matsuoka, Y., and Nasuda, S. (2004) Durum wheat as a candidate for the unknown female progenitor of bread wheat: an empirical study with a highly fertile F1 hybrid with *Aegilops tauschii* Coss. *Theoretical and Applied Genetics* **109**, 1710-1717.

Michaels, S.D., and Amasino, R.M. (2001) Loss of *FLOWERING LOCUS C* activity eliminates the late-flowering phenotype of *FRIGIDA* and autonomous pathway mutations but not responsiveness to vernalization. *Plant Cell* **13**, 935-941.

Michaels, S.D., and Amasino, R.M. (1999) *FLOWERING LOCUS C* encodes a novel MADS domain protein that acts as a repressor of flowering. *Plant Cell* **11**, 949-956.

Minorsky, P.V. (2002) The Hot and the Classic. *Plant Physiology* **129**, 5-6.

Moon, J., Suh, S.S., Lee, H., Choi, K.R., Hong, C.B., Paek, N.C., Kim, S.G. and Lee, I. (2003) The *SOC1* MADS-box gene integrates vernalization and gibberellin signals for flowering in *Arabidopsis*. *The Plant Journal* **35**, 613-623.

Nath, J., McNay, J.W., Paroda, C.M., and Gulati, S.C. (1983) Implication of *Triticum searsii* as the B-genome donor to wheat using DNA hybridizations. *Biochemical Genetics* **21**, 745-760.

Okamuro, J.K., den Boer, B.G.W., Lotys-Prass, C., Szeto, W., and Jofuku, K.D. (1996) Flowers into shoots: Photo and hormonal control of a meristem identity switch in *Arabidopsis*. *Proceedings of the National Academy of Sciences USA* **93**, 13831–13836.

Page, T., Macknight, R., Yang, C.H. and Dean, C. (1999) Genetic interactions of the Arabidopsis flowering time gene *FCA*, with genes regulating floral initiation. *Plant Journal* **17**, 231–239.

Parcy, F. (2005) Flowering: A Time For Integration. *International Journal of Developmental Biology* **49**, 585-593.

Parcy, F., Bomblies, K., and Weigel, D. (2002) Interaction of *LEAFY*, *AGAMOUS* and *TERMINAL FLOWER1* in maintaining floral meristem identity in *Arabidopsis*. *Development* **129**, 2519-2527.

Peng, J., Richards, D.E., Hartley, N.M., Murphy, G.P., Devos, K.M., Flintham, J.E., Beales, J., Fish, L.J., Worland, A.J., Pelica, F., Sudhakar, D., Christou, P., Snape, J.W., Gale, M.D. and Harberd, N.P. (1999) 'Green revolution' genes encode mutant gibberellin response modulators. *Nature* **400**, 256-261.

Prasil, I.T., Prasilova, P. and Pankova, K. (2004) Relationships among vernalization, shoot apex development and frost tolerance in wheat. *Annals of Botany* **94**, 413-418.

Quesada, V., Macknight, R., Dean, C., and Simpson, G. G. (2003) Autoregulation of *FCA* pre-mRNA processing controls *Arabidopsis* flowering time. *The European Molecular Biology Organization Journal* **22**, 3142–3152.

Quesada, V., Dean, C., and Simpson, G.G. (2005) Regulated RNA processing in the control of Arabidopsis flowering. *International Journal of Developmental Biology* **49**,773-80

Razem, F.A., El-Kereamy, A., Abrams, S.R., and Hill, R.D. (2006) The RNA-binding protein *FCA* is an abscisic acid receptor. *Nature* **439**, 290-294.

Razem, F.A., Luo, M., Liu, J.H., Abrams, S.R. and Hill, R.D. (2004) Purification and characterization of a Barley aleurone abscisic acid-binding protein. *Journal of Biological Chemistry* **279**, 9922-9929.

Rouse, D.T., Sheldon, C.C., Bagnall, D. J., Peacock, W.J. and Dennis, E.S. (2002) *FLC*, a repressor of flowering, is regulated by genes in different inductive pathways. *The Plant Journal* **29**, 183-191.

Samach, A., and Coupland, G. (2000) Time measurement and the control of flowering in plants. *Bioessays* **22**, 38-47.

Sandhu, D., and Gill, K.S. (2002) Gene-containing regions of wheat and the other grass genomes. *Plant Physiology* **128**, 803-811.

Sarma, R.N., Fish, L., Gill, B.S., and Snape, J.W. (2000) Physical characterization of the homoeologous group 5 chromosomes of wheat in terms of rice linkage blocks, and physical mapping of some important genes. *Genome* **43**, 191-198.

Sarma, R.N., Gill, B.S., Sasaki, T., Galiba, G., Sutka, J., Laurie, D.A., and Snape, J.W. (1998) Comparative mapping of the wheat chromosome 5A Vrn-A1 region with rice and its relationship to QTL for flowering time. *Theoretical and Applied Genetics* **97**, 103-109.

Sarnowski, T.J., Swiezewski, S., Pawlikowska, K., Kaczanowski, S., and Jerzmanowski, A. (2002) AtSWI3B, an *Arabidopsis* homolog of SWI3, a core subunit of yeast Swi/Snf chromatin remodeling complex, interacts with FCA, a regulator of flowering time. *Nucleic Acids Research* **30**, 3412-3421.

Sarnowski, T.J., Swiezewski, S., Rios, G., Pawlikowska, K., Kozbial, M., Kozbial, P., Kuras, M., Koncz, C., and Jerzmanowski, A. (2003) Functional characterization of *Arabidopsis AtSWI3* genes encoding homologs of a core subunit of yeast SWI/SNF chromatin remodeling complex. *European Journal of Biochemistry* **1** Supplement, P12-14.

Schleinkofer, K., Wiedemann, U., Otte, L., Wang, T., Krause, G., Oschkinat, H. and Wade, R.C. (2004) Comparative structural and energetic analysis of WW Domain-Peptide interactions. *Journal of Molecular Biology* **344**, 865-881.

Sheldon, C.C., Burn, J.E., Perez, P.P., Metzger, J., Edwards, J.A., Peacock, W.J. and Dennis, E.S. (1999) The *FLF* MADS-box gene: a repressor of flowering in *Arabidopsis* regulated by vernalization and methylation. *Plant Cell* **11**, 445-458.

Sheldon, C.C., Finnegan, E.J., Rouse, D.T., Tadege, M., Bagnall, D.J., Helliwell, C.A., Peacock, W.J. and Dennis, E.S. (2000a) The control of flowering by vernalization. *Current Opinion in Plant Biology* **3**, 418-422.

Sheldon, C.C., Rouse, D.T., Finnegan, E.J., Peacock, W.J. and Dennis, E.S. (2000b) The molecular basis of vernalization: The central role of *FLOWERING LOCUS C* (*FLC*). *Proceedings of the National Academy of Sciences USA* **97**, 3753-3758.

Shindo, C., Aranzana, M.J., Lister, C., Baxter, C., Nicholls, C., Nordborg, M. and Dean, C. (2005) Role of *FRIGIDA* and *FLOWERING LOCUS C* in determining variation in flowering time of *Arabidopsis*. *Plant Physiology* **138**, 1163-1173.

Simpson, G.G., Dijkwel, P.P., Quesada, V., Henderson, I. and Dean, C. (2003) FY is an RNA 3' prime end-processing factor that interacts with FCA to control the *Arabidopsis* floral transition. *Cell* **113**, 777-787.

Snape, J.W., Butterworth, K., Whitechurch, E., and Worland, A.J. (2001) Waiting for fine times: genetics of flowering time in wheat. *Euphytica* **119**, 185-190

Sung, S., and Amasino, R.M. (2004) Vernalization and epigenetics: how plants remember winter. *Current Opinion in Plant Biology* **7**, 4-10.

Thompson, J.P., and Nath, J. (1986) Elucidation of the B-genome donor to *Triticum turgidum* by unique- and repeated-sequence DNA hybridizations. *Biochemical Genetics* **24**, 39-50.

Toth, B., Galiba, G., Feher, E., Sutka, J., and Snape, J.W. (2003) Mapping genes affecting flowering time and frost resistance on chromosome 5B of wheat. *Theoretical and Applied Genetics* **107**, 509-514.

Yan, L., Loukoianov, A., Tranquilli, G., Helguera, M., Fahima, T., and Dubcovsky, J. (2003) Positional cloning of the wheat vernalization gene *VRN1*. *Proceedings of the National Academy of Sciences USA* **100**, 6263-6268.

Yano, M., Katayose, Y., Ashikari, M., Yamanouchi, U., Monna, L., Fuse, T., Baba, T., Yamamoto, K., Umehara, Y., Nagamura, Y., and Sasaki, T. (2000) *Hd1*, a major photoperiod sensitivity quantitative trait locus in rice, is closely related to the *Arabidopsis* flowering time gene *CONSTANS*. *Plant Cell* **12**, 2473-2484.

Zhang, D., Choi, D.W., Wanamaker, S., Fenton, R.D., Chin, A., Malatrasi, M., Turuspekov, Y., Walia, H., Akhunov, E.D., Kianian, P., Otto, C., Simons, K., Deal, K.R., Echenique, V., Stamova, B., Ross, K., Butler, G.E., Strader, L., Verhey, S.D., Johnson, R., Altenbach, S., Kothari, K., Tanaka, C., Shah, M.M., Laudencia-Chingcuanco, D., Han, P., Miller, R.E., Crossman, C.C., Chao, S., Lazo, G.R., Klueva, N., Gustafson, J.P., Kianian, S.F., Dubcovsky, J., Walker-Simmons, M.K., Gill, K.S., Dvorak, J., Anderson, O.D., Sorrells, M.E., McGuire, P.E., Qualset, C.O., Nguyen, H.T., and Close, T.J. (2004) Construction and evaluation of cDNA libraries for large-scale expressed sequence tag sequencing in wheat (*Triticum aestivum* L.). *Genetics* **168**, 595-608.

APPENDIX 1

Analysis of the Flowering Transition in T1 Progeny Germinated on Half-Strength MS Media Plates

While the major study for this experiment involved the analysis of numerous *WFCA-A* transgenic T1 progeny grown directly in soil, an additional 47 *WFCA-A* T1 plants were germinated on half-strength (½) MS Media containing 50 μg ml^{-1} kanamycin. The initial aim of this experiment was to check for the transgene in the T1 progeny, as only those plants containing a successful insertion would survive kanamycin selection. However, upon confirmation of survival on kanamycin, the decision was made to check the flowering behaviour of these plants. Although the proper controls were not used for this study, it was thought that some insights into the effects of *WFCA* expression in the T1 plants might be gained by transferring these plants into soil and allowing them to grow.

Ler-0 and *fca-1* plants were germinated on non-selective ½ MS Media, and therefore do not provide comparable controls due to the absence of kanamycin in the media and its potential effects on these plants. Both transgenic and parental plants were transferred to 36-cell trays containing Rediearth growth media and grown under LD conditions until bolting. The number of days to bolting and the rosette FLN was recorded using the same criteria for the soil-grown plants (Table A1).

Table A1. Analysis of Media-Transferred T1 Progeny and Flowering Controls.

Background	# Plants	Min FLN	Max FLN	Avg FLN	Avg DtB[a]
Ler-0 control	11	6	9	7.6 ± 1.2	25.2 ± 0.6
fca-1 control	10	7	11	9.6 ± 2.0	25.6 ± 1.0
WFCA-A T1	47	6	22+	10.8 ± 2.4	28.0 ± 3.0

a) DtB = Days to Bolting.

Unlike the *WFCA-A* T1 plants initially grown in soil, the media-transferred *WFCA-A* T1 plants showed more variation in their flowering behaviour, as indicated by the relatively small differences between the Ler-0 and *fca-1* control plants. However, in this situation, the media-transferred T1 progeny displayed the most significant variations in FLN, as well as several phenotypic variations, including some plants in which the first three rosette leaves produced were oversized. One plant also produced over 22 rosette leaves; however it died before producing seeds (Figure A1).

One-way ANOVA on the FLN of the media-transferred T1 progeny shows that while the difference between the T1 progeny FLN and the Ler-0 "control" FLN was significant, the differences between the T1 progeny FLN and Ler-0 "control" FLN with the *fca-1* "control" FLN were not significant. Since the control Final Leaf Numbers were not significantly different from each other, it raises questions as to the use of these data. The data is included here for archival purposes only.

It is important to note that the sample size and lack of true controls in this trial raise concerns with any conclusions drawn from this data. With this caution, the variability between the results observed in the soil-grown T1 progeny in comparison to the media- transferred T1 plants may be attributed to several different factors. First, the tray size was significantly different in both cases. Soil-grown T1 plants were sown into

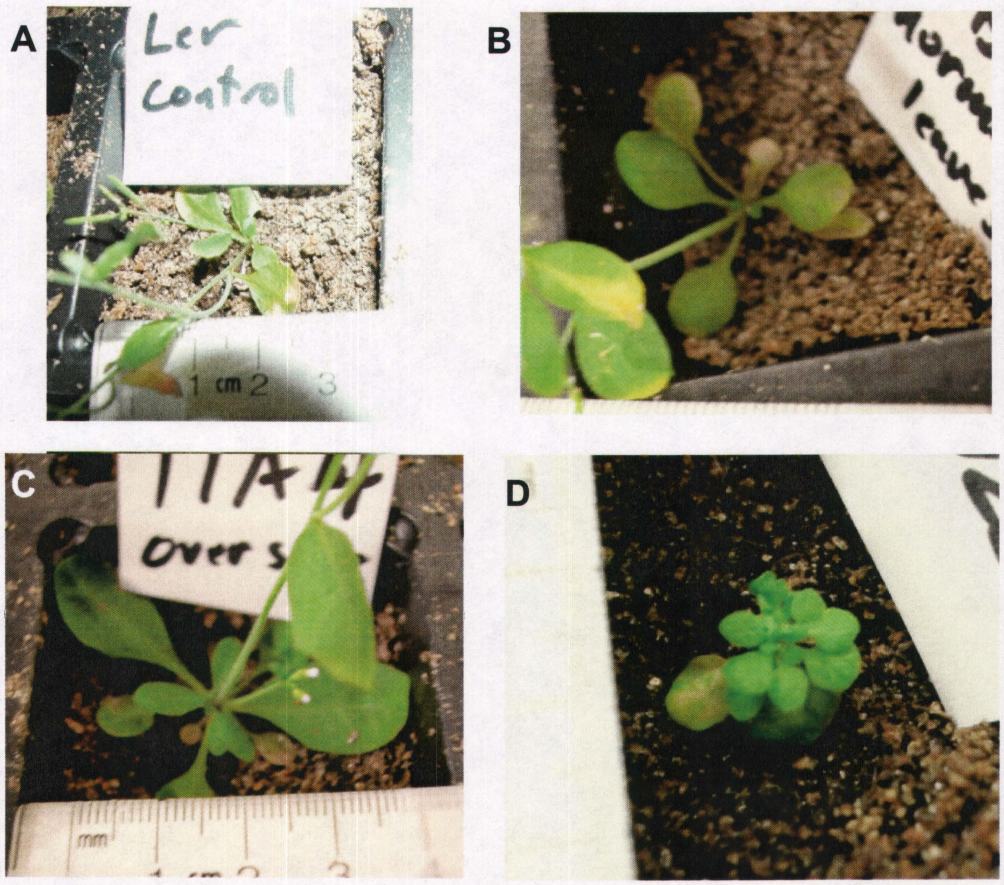

Figure A1. Morphological Variations in T1 Media-Transferred Progeny.

Unlike the soil-grown T1 progeny, media-transferred progeny displayed some morphological variation, including an extremely high FLN or the initial 3 rosette leaves displaying a significant increase in their surface area.
A) Ler-0 control. B) Normal T1 plant. C) T1 plant displaying oversized leaf triad.
D) T1 plant with a FLN of 22.

72-cell trays, with each cell measuring 1½" x 1½" and a depth of ~2". In comparison, media-transferred T1 plants were grown in 36-cell trays, with each cell measuring 2¼" x 2", with a depth of ~2". Based on these measurements, the media-transferred plants had over twice the amount of growing area of the soil-grown plants. Whereas the soil-grown plants were contained within smaller cells to accommodate a greater number of plants,

the smaller growth space might have curtailed plant growth.

Second, the presence of kanamycin in the media may have affected the T1 plants, possibly through providing additional stress on the young plants. Similarly, since the exact location of transgene insertion was not determined, if the area of insertion is sensitive to kanamycin, or if the insertion affected the plant's ability to deal with stress, it would account for the discrepancy in FLN between the soil-grown and media-transferred plants. Third, the process of transferring plants from a media plate to soil, although done carefully, involves the introduction of some stress to the plants, particularly in regards to root damage. Transgenic plants coping with the production of a foreign gene product may have an altered response to wounding or root damage, and this would contribute to the variability between phenotypes seen in the two sets of transgenic trials. Additional replicates of the T1 generation and the appropriate controls will be required to draw any conclusions.

APPENDIX 2

Generation of Polyclonal WFCA Antibodies

Originally, the objective was to generate WFCA-antibodies for use in detection of transgenically expressed WFCA protein. Unfortunately, due to the time delays in this project, Western analysis of transgenic plants was abandoned. Here, the production of the polyclonal antibodies is documented.

Production of WFCA protein required a low temperature induction (24°C) in LB media containing 0.66M sorbitol and 2.5 mM betaine. Sonicated cell extracts were run on a 7.5% SDS-PAGE gel to determine the localization of expressed WFCA in soluble or insoluble fractions of the protein extract (Figure A2). Expressed protein was primarily seen in the insoluble fraction. The WFCA polypeptide was isolated from the insoluble fraction of induced BL21 (DE3) cells and purified using His-Bind Quick 900 cartridges (Novagen, EMD Biosciences, Inc./ Merck KGaA, Darmstadt, Germany). Purified protein was run on 7.5% SDS-PAGE gels and stained with $CuCl_2$ for 30 min. Protein bands were isolated using a sharp scalpel, lyophilized in a vacuum-dryer, and finely ground using mortar and pestle. The protein was vacuum-desiccated, ground under liquid nitrogen, and provided to Vaccine and Infectious Disease Organization (Saskatoon, SK) for raising polyclonal antibodies in rabbits. Rabbits had been fed canola meal to reduce the incidence of cross-reactivity to wheat protein present in a normal diet.

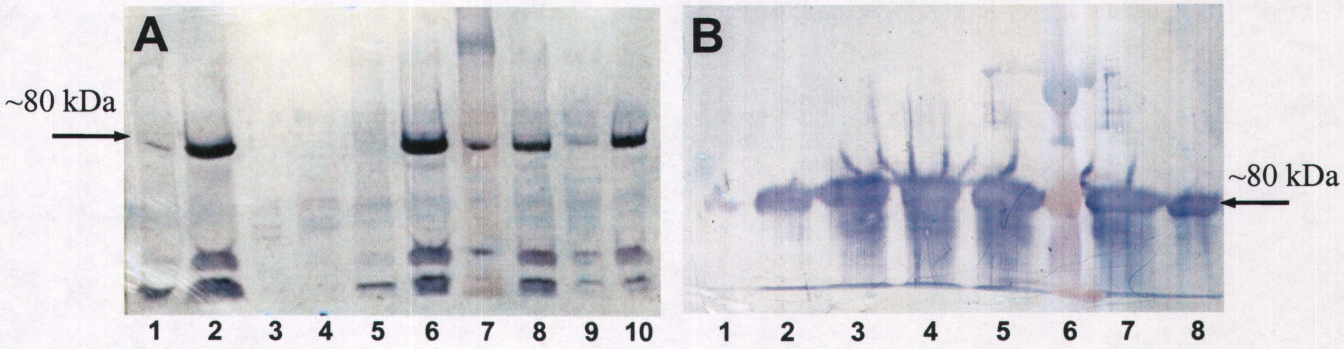

Figure A2. Western Blots of Expressed WFCA.
All gels were 7.5% SDS-PAGE blotted onto PVDF membrane.
A) Induction of recombinant WFCA cloned into vector pET-28a (see Materials and Methods) to determine protein localization. Protein was reacted with His-Tag antibody which reacts with the His fusion tag at the N-terminal of the E. coli expressed WFCA polypeptide. WFCA was localized to the insoluble fraction.
Lanes: 1 = uninduced total cell protein, 2 = induced total cell protein, 3 = soluble fraction from uninduced cells, 4 = soluble fraction from induced cells, 5 = insoluble fraction from uninduced cells, 6 = insoluble fraction from induced cells, 7 = ladder, 8 = total cell protein from induced cells (2^{nd} culture), 9 = soluble fraction from induced cells (2^{nd} culture), 10 = insoluble fraction from induced cells (2^{nd} culture).
B) Expressed recombinant WFCA was purified using His-tag columns and run in varying concentrations (indicated) on a 7.5% polyacrylamide gel under denaturing conditions (SDS-PAGE). The predicted size for WFCA is ~80 kDa. The membrane was reacted with antiserum from the 3^{rd} bleed of rabbits injected with purified WFCA.
Lanes: 1: blank, 2: WFCA column flowthrough, 3: 80 ug WFCA, 4: WFCA overflow, 5: 50 ug WFCA, 6: ladder, 7: 34 ug WFCA, 8: 17 ug WFCA.

Blood samples obtained from VIDO were clotted at 37°C for 1 hour, then placed at 4°C overnight to allow blood clots to contract. Serum was extracted by centrifuging the sample at 10,000g at 4°C for 10 minutes in sterile, baked Corex tubes. Serum aliquots were kept at 4°C for immediate use, and the remainder of the sample was stored at -20°C.

To test the anti-serum for WFCA-binding activity, *E. coli* expressed WFCA was loaded in varying concentrations and run on a 7.5% SDS-PAGE gel. The gel was blotted

onto a PVDF membrane and reacted for 20 hours with antibodies isolated from the 3rd bleed.