"Enjoy failure and learn from it. You can never learn from success."
— James Dyson

University of Alberta

TtABCC3 encodes an ABC transporter linked to cadmium accumulation in durum wheat grain (*Triticum turgidum* L. var. *durum*)

by

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Abstract

Health concerns associated with cadmium (Cd) in the grain of durum wheat have prompted the development of low grain-Cd cultivars. Differences in Cd accumulation between cultivars have been attributed to retention of Cd in the roots of low-Cd cultivars. However, our understanding of the molecular mechanism for root retention remains unknown. Microarray analysis of root tissue from low-Cd and high-Cd accumulating near-isogenic lines identified *TtABCC3* as a gene with higher expression in the low-Cd isoline. The full-length coding region of *TtABCC3* was isolated and sequencing identified two non-homeologous genes (*TtABCC3α* and *TtABCC3β*) corresponding to the A and B genomes. Both genes were unable to restore Cd tolerance in a Cd-hypersensitive yeast strain defective in Cd transport across the tonoplast, suggesting that TtABCC3 is not involved in sequestration of Cd in the vacuole. Nonetheless, this experimental approach provides a framework for characterizing other genes identified from the microarrays.

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Early on in my project I came across numerous difficulties while attempting to isolate *TtABCC3*. I still remember the day that Gary Ritzel showed me the RCA-RACE method, and we worked together to resolve this issue. I cannot thank him enough for his help and patience. He is truly a skilled scientist and it was an honour to work alongside him. I would also like to thank James Maclagan for his assistance with other molecular biology techniques. I wish to thank the staff at the Molecular Biology Service Unit for their help with sequencing, instrument use and other general techniques. Without these services my research would have not progressed in a timely-fashion.

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List of Symbols, Nomenclature, or Abbreviations

ABC, ATP-binding cassette

ABCC, ATP-binding cassette subfamily C

Ag, gold

Ar, arsenic

BKG, background

BLAST, basic local alignment and search tool

Bn-NCC1, *Brassica napus* nonfluorescent chlorophyll catabolite 1

BPT1, bile pigment transporter 1

CAX, cation-exchange transporter

Cd, cadmium

cDNA, complimentary DNA

Cu, copper

DFCI, Dana-Farber Cancer Institute

DMSO, dimethyl sulfoxide

DNP-GS - *S*-(2,4-dinitrophenyl)glutathione

EC, electrical conductivity

EDTA, ethylenediaminetetraacetic acid

FDR, false discovery rate

Fe, iron

GF-AAS, graphite furnace atomic absorption spectroscopy

GFP, green fluorescent protein

GS_2-Cd, bis(glutathionato)cadmium

GSH, glutathione (reduced)

GS-X, glutathione conjugate

GUS, β-glucuronidase

HEDTA, N-(2-hydroxyethyl)ethylenediaminetriacetic acid

Hg, mercury

HMA, heavy metal transporting P-type ATPases

HMT1, heavy metal tolerance factor 1

iPCR, inverse polymerase chain reaction

LCT1, low-affinity cation transporter 1

MES, 2-(N-morpholino)ethanesulfonic

MRP, multidrug resistance-associated protein

NBF, nucleotide-binding fold

NCBI, National Center for Biotechnology Information

NIL, near-isogenic line

NIST, National Institute of Standards and Technology

NTE, N-terminal extension

OD, optical density

Pb, lead

PC, phytochelatin

PCR, polymerase chain reaction

PCS, phytochelatin synthase

PTA, Plant Transcript Assemblies

QC, quality control

qRT-PCR, quantitative reverse transcription-polymerase chain reaction

RACE, rapid amplification of cDNA ends

RCA-RACE, rolling circle amplification-rapid amplification of cDNA ends

RO, reverse osmosis

RQ, risk quotient

RT-PCR, reverse transcription-polymerase chain reaction

SAM, significance analysis of microarrays

SC, synthetic complete

SC-HIS, synthetic complete without histidine

SDS, sodium dodecyl sulfate

*Ta*GI, *Triticum aestivum* gene index

TC, tentative consensus

TIGR, The Institute for Genomic Research

TMD, transmembrane domain

TtABCC3, *Triticum turgidum* ATP-binding cassette subfamily C 3

UID, unique identification number

UTR, untranslated region

YCF1, yeast cadmium factor 1

YPD, yeast peptone dextrose

ZIP, ZRT-/IRT-like protein

Zn, zinc

1. Introduction

1.1. Cadmium

Cadmium (Cd) is a toxic metal that is found ubiquitously in the environment. Baker (1990) commented that Cd is never found as an isolated metal (e.g. Cd ore), but rather as a 'guest' metal from lead/zinc mineralization. Natural soils generally contain 0.1 to 2.0 mg Cd kg^{-1} (McLaughlin *et al.*, 1999). However, much higher Cd concentrations have been recorded in specific soil associations, such as the Monterey shale soils in coastal southern California, where Cd concentrations are up to 30 mg kg^{-1} (Page *et al.*, 1981).

1.1.1. Sources of Cadmium in Agricultural Soils

Agricultural systems can have elevated levels of Cd due to addition of Cd from phosphate fertilizers, sewage sludge, manure, and, to a lesser extent, irrigation water and atmospheric deposition (Alloway and Steinnes, 1999). Inputs of Cd to agricultural lands often exceed the output from crops and drainage (leaching), thus Cd concentrations are gradually increasing (McLaughlin *et al.*, 1999).

Phosphate fertilizers are derived from phosphate rocks that have varying levels of Cd, resulting in Cd contents that can reach in excess of 300 mg kg^{-1} (Alloway and Steinnes, 1999). The potential long-term risk of Cd input from phosphate fertilizers for 15 locations in Canada was investigated by Grant and Sheppard (2008). Each location was assigned a risk quotient (RQ) that was calculated by dividing the total soil Cd content by the Canadian Council of Ministers of the Environment guideline of 1.4 mg kg^{-1}. Soil samples considered to be safe had an $RQ \leq 1$. Mean RQ values ranged from

0.04 to 2.3, indicating some sites exceed the acceptable soil Cd levels and therefore need to be managed to minimize Cd inputs. Short-term field experiments have also demonstrated that phosphate fertilizers contribute to soil Cd content. Lambert *et al.* (2007) showed that application of phosphate fertilizer over a 3-year period increased soil Cd proportionally to the fertilizer Cd content and the rate of application. It should be noted that no crops were grown on the experimental fields during the 3-year period. Plant growth often reduces Cd remaining in the soil after each application.

The addition of sewage sludge to farmlands has been perceived as being an effective end-use of sludge by government municipalities. However, sludge often contains toxic levels of metals that can subsequently accumulate in crops (McBride, 2003). Gutenmann *et al.* (1982) found tobacco grown on fields amended with sludge had 20-fold higher leaf Cd concentrations than tobacco grown on non-amended fields.

1.1.2. Uptake of Soil Cadmium by Plants

Root uptake of Cd from the soil is the primary route for accumulation in plants. The bioavailability of Cd^{2+} in soil is higher than other toxic metals such as mercury (Hg), lead (Pb), and arsenic (As), and is therefore taken up at a greater rate into the plant (Clemens, 2006). As a result, soil Cd content often correlates with Cd concentrations in the plant, and poses a greater risk for crop contamination than other metals.

Numerous factors affect the bioavailability of Cd^{2+} in soil, including total Cd content, pH, total organic matter content, salinity, cation exchange capacity, and the presence of other plant nutrients (Marschner, 1995; McLaughlin *et al.*, 1999; Greger, 2004). Soil Cd content and pH seem to play a major role in controlling Cd bioavailability.

Soils enriched in iron oxides have nearly all Cd bound to sorption sites at pH 6, but little Cd is adsorbed below pH 4.5 (Wagner, 1993). Increasing soil pH leads to greater metal adsorption because there is an increase in net negative charge on the soil surface (Naidu *et al.* (1997). Wagner (1993) estimated that acidic soils would have twice the Cd^{2+} concentration in solution than neutral soils, thus increasing Cd content in plants. Wang *et al.* (2006) found total shoot Cd in *Thlaspi caerulescens* increased 61% when the soil pH was reduced from 6.9 to 5.3. Plant roots themselves can also modify the rhizosphere by releasing organic anions, such as citrate, malate and acetate, which increases localized metal concentrations (Jones, 1998). Qin *et al.* (2004) found organic ligands play a dominant role in the mobilization of metals [Cd, copper (Cu), and Pb] from soils. This effect was independent of pH, indicating these ligands can manipulate the soil-metal equilibrium of the rhizosphere.

Plant accumulation of Cd from agricultural soils does not typically reach sufficient levels to elicit a phytotoxic response in crops. At toxic concentrations, plants show symptoms of chlorosis, necrosis, leaf rolling, and stunted root growth (Sanità di Toppi and Gabbrielli, 1999). Although the mechanism of Cd toxicity is not well defined, Cd is known to disrupt photosynthesis, stomatal opening, transpiration, water balance and nutrient uptake (Das *et al.*, 1997; Sanità di Toppi and Gabbrielli, 1999; Baryla *et al.*, 2001; Benavides *et al.*, 2005). Cadmium can also generate reactive oxygen species (ROS), which can interfere with plant metabolic processes, such as respiration and photosynthesis (Asada and Takahashi, 1987). Unlike the transition elements Cu and iron (Fe), Cd^{2+} is a redox-inactive metal; ROS levels are increased indirectly by reducing the

glutathione (GSH) pool and inhibiting antioxidant proteins by blocking functional thiol groups (Schützendübel and Polle, 2002).

1.1.3. Human Health Concerns with Cadmium Accumulation

Consumption of crops grown on agricultural soils is the primary source of dietary Cd for humans, since most crops accumulate Cd without showing symptoms of phytotoxicity (Wagner, 1993; McLaughlin *et al.*, 1999). Plant consumption may account for 66% to 70% of dietary Cd exposure (Ryan *et al.*, 1982; Satarug and Moore, 2004). Intake of Cd is even higher for those who smoke, as tobacco accumulates higher Cd content in the leaf than most crops (Lugon-Moulin *et al.*, 2004).

Cadmium accumulates primarily in the liver and kidney, and also in the lungs of smokers. Studies on autopsies of individuals not exposed to Cd in their workplace (representing the general population) found levels of Cd in kidney and liver increased with age (Satarug *et al.*, 2003). Rates of accumulation were faster in the kidney. Ryan *et al.* (1982) calculated that the excretion rate of Cd is only 0.005% of body burden. Furthermore, Cd has a predicted half-life of more than 20 years in the body (Elinder *et al.*, 1976), suggesting continuous ingestion of food would increase Cd body burden with age. Chronic exposure of Cd has been linked to renal dysfunction and osteoporosis (Satarug and Moore, 2004), and Cd is classified as a human carcinogen (IARC, 1993).

1.1.4. Strategies to Reduce Cadmium Content in Crops

The potential health concerns of long-term Cd exposure have prompted changes in agronomic management practices to reduce soil-plant transfer of Cd (McLaughlin *et*

al., 1999). Current methods include reducing fertilizer use, the use of low-Cd fertilizers, liming, the addition of zinc (Zn), reducing soil salinity, and seasonal crop rotation. Among these methods, reducing Cd content in commercial fertilizers and/or the amount applied to crop fields might be the most effective long term strategy for reducing soil Cd concentrations (McLaughlin *et al.*, 1996). Liming, which decreases Cd solubility by neutralizing soil pH, has been effective in lowering plant Cd uptake in potted experiments (Andersson and Nilsson, 1974; Williams and David, 1976; Eriksson, 1989; Maier *et al.*, 2002; Tlustoš *et al.*, 2006), but some researchers have been unable to reproduce these results under field conditions (Andersson and Siman, 1991; Li *et al.*, 1996a; Maier *et al.*, 1996). These inconsistencies were attributed to insufficient changes in soil pH (Li *et al.*, 1996a), limited diffusion of lime beyond the zone of application (Maier *et al.*, 1996), and competitive interactions of calcium (Ca^{2+}) ions with Cd^{2+} adsorption sites (Christensen, 1984). Application of Zn has decreased Cd content in crops, mostly when soils are Zn-deficient (Oliver *et al.*, 1994; Choudhary *et al.*, 1995). Elevated soil salinity can increase the mobilization of Cd by forming Cl_2Cd complexes, leading to increased plant uptake (McLaughlin *et al.*, 1997); however, lowering salt concentrations in the soil often requires leaching of growing fields or improving the quality of irrigation water (McLaughlin *et al.*, 1999).

Overall, many of these strategies are only temporary solutions to reducing soil-plant transfer of Cd, and are often ineffective and/or expensive to implement. In addition, the complex chemistry of soil-Cd interactions can vary greatly among agricultural soils and a simple method that can be successfully applied to all soil types likely does not exist.

1.2. Accumulation of Cadmium in Crops

Accumulation of Cd occurs readily in plants. The amount of Cd accumulated and how the Cd is distributed within the plant differs greatly between crops. The distribution of Cd within plants is affected by root morphology, root uptake, root-to-shoot translocation, phloem mobilization, and subcellular storage (Greger, 2004). Rates of accumulation and relative tissue content can also vary throughout the development of the plant. Generally, concentrations of Cd in edible tissue are leaf > fruit, fibrous root > grain and seed (Wagner, 1993). Older leaves contain higher Cd content than younger leaves. Leafy vegetables, such as spinach and lettuce, are classified as high Cd accumulating plants (Kuboi *et al.*, 1986), but are not considered a current health risk because they have low prevalence in the human diet.

Natural variation of Cd accumulation also occurs among cultivars within species, suggesting that the development of cultivars selected for low Cd concentrations is a viable approach to reduce Cd input into the food chain (Grant *et al.*, 2008). Such variation has been observed for several crop species including maize (*Zea mays* L.; Hinesly *et al.*, 1978), barley (*Hordeum vulgare* L.; Chang *et al.*, 1982; Chen *et al.*, 2007), flax (*Linum usitatissimum* L.; Grant *et al.*, 2000; Hocking and McLaughlin, 2000), oat (*Avena sativa* L.; Tanhuanpää *et al.*, 2007), potato (*Solanum tuberosum* L.; McLaughlin *et al.*, 1994; Dunbar *et al.*, 2003), rice (*Oryza sativa* L.; Arao and Ae, 2003; Arao and Ishikawa, 2006), soybean (*Glycine max* L.; Arao *et al.*, 2003; Ishikawa *et al.*, 2005; Arao and Ishikawa, 2006), bread wheat (Triticum aestivium L.; Kjellström *et al.*, 1975; Andersson and Pettersson, 1981; Gray *et al.*, 2001; Stolt *et al.*, 2006), and durum wheat

(*Triticum turgidum* L. var. *durum*; Clarke *et al.*, 1997b; Clarke *et al.*, 1997a; Li *et al.*, 1997a; Clarke *et al.*, 2002; Greger and Löfstedt, 2004; Stolt *et al.*, 2006).

Often Cd content in edible tissues can vary by several-fold among cultivars within a species. Chen *et al.* (2007) reported grain Cd concentrations among 600 barley genotypes ranged from 0 (undetectable) to 1.21 mg kg^{-1}, with a mean concentration of 0.16 mg kg^{-1}. Moreover, nearly half of the grain samples exceeded acceptable levels of Cd for cereal grains, indicating the need for development of low-Cd cultivars. Rice grown on soils amended with sludge accumulated between 0.06 and 0.99 mg Cd kg^{-1}, and these variations were explained by differences in root uptake (He *et al.*, 2006). Screening of over 2,700 accessions of flax showed seed Cd ranged from 0.27 to 3.60 mg kg^{-1}, indicating a large variation in genotypes within the species (Hammond *et al.*, 1999).

Variation for Cd content within each crop species does indicate low-Cd varieties can be generated, but this process is laborious and costly (Grant *et al.*, 2008). Initially, breeders must find genetic variation in Cd concentration (in the desired tissue) of the current germplasm and then understand how the low-Cd trait is inherited. These low-Cd traits must be integrated into modern cultivars that possess desirable traits including high yield, disease resistance, and other quality traits. Recently, low-Cd cultivars for sunflower (Miller *et al.*, 2006) and durum wheat (Clarke *et al.*, 2005, 2006) have been registered, indicating breeding programs can provide a means to lower Cd concentrations in crop species.

1.3. Accumulation of Cadmium in Durum Wheat (*Triticum turgidum* L. var. *durum*)

Durum wheat has a harder grain than bread wheat, and the grain is mostly used in pastas. Durum wheat has a greater propensity to accumulate Cd in the grain than bread wheat (Meyer *et al.*, 1982), which could be due to differences in root uptake and/or tissue partitioning of Cd in the plant. Cadmium content in durum wheat grain is usually lower than edible tissues of other crops, yet durum wheat still serves as a major source of Cd for the human diet because it is consumed at a greater frequency.

Given the potential health risks associated with consumption of foodstuffs contaminated with Cd, the Codex Alimentarius Commission is considering standards for maximum Cd levels in a variety of internationally traded food commodities (Codex Alimentarius Commission, 2005). A limit of 0.2 mg kg^{-1} has been proposed for wheat grain. This limit raises a potential economic risk for Canada, as durum wheat grain can exceed this level. Surveys of durum wheat grown in the Canadian prairies measured grain Cd between 0.1 to 0.5 mg kg^{-1} (Garrett *et al.*, 1999) and 0.025 to 0.359 mg kg^{-1} (Norvell *et al.*, 2000). Grain Cd concentrations of a variety of durum wheat cultivars from South Dakota ranged from 0.13 to 0.25 mg kg^{-1}, and averaged 0.22 mg kg^{-1} (Erdman and Moul, 1982). Furthermore, with Canada being among the world's leading producers of durum wheat (PECAD, 2005), ensuring marketability in countries with current Cd limits (e.g. Sweden; Olsson *et al.*, 2005) is an economic necessity. Therefore, the development of low grain-Cd cultivars is an important priority to ensure long-term access to foreign markets.

A collection of durum wheat genotypes varying in grain Cd accumulation has been produced using classical breeding techniques by Agriculture and Agri-Food Canada, including low-Cd and high-Cd accumulating, near-isogenic lines (NILs; Clarke *et al.*, 1997b). This research culminated in the recent registration of a low-Cd durum wheat cultivar, 'Strongfield' (Clarke *et al.*, 2005, 2006), that may alleviate some of the concerns related to the international Cd limits. Beyond development of low-Cd germplasm, breeding programs have not increased our understanding of the mechanisms regulating Cd grain accumulation. Insight into the mechanistic basis for Cd accumulation in durum wheat could aid in the development of low-Cd cultivars for other crops, such as rice, potato, and flax (McLaughlin *et al.*, 1999).

Current research has focused on the physiological processes regulating accumulation of Cd in the grain of durum wheat, which has primarily been attributed to increased root retention in the low-Cd cultivars and to a lesser extent remobilization during grain development.

1.3.1. Root-to-Shoot Translocation of Cadmium

Studies utilizing low-Cd and high-Cd accumulating NILs suggest restricted root-to-shoot translocation through the xylem is a critical step in regulating grain Cd accumulation (Harris and Taylor, 2001, 2004). The low-Cd isoline retained nearly two-fold more Cd in the roots, even though total plant levels remained the same for both NILs (Harris and Taylor, 2004). Differences in accumulation of Cd in grain between low-Cd and high-Cd cultivars were also attributed to reduced translocation in the low-Cd cultivar during flowering (Chan and Hale, 2004). Greger and Löfstedt (2004) reported that

distribution of Cd concentrations among various cultivars of durum wheat grown in solution culture was explained by differences in translocation from the root to shoot, and not from root uptake. The importance of the roots in controlling shoot Cd concentrations has also been shown in *Nicotiana* (tobacco) by reciprocal grafting experiments between low-Cd and high-Cd accumulating species (Wagner *et al.*, 1988). Stem grafts of low-Cd shoots from *N. rustica* onto the roots of the high-Cd *N. tabacum* resulted in accumulation of Cd in shoots that was typical of high-Cd species. Sugiyama *et al.* (2007) also found that seed Cd concentrations in soybean was dictated by the rootstock cultivar in grafting studies.

Differential levels of Cd in the grain of durum wheat are not due to total plant accumulation, but rather partitioning of Cd within the plant. Increased retention of Cd in roots suggests a mechanism of sequestration within root cells or exclusion during xylem loading. The Cd content of xylem sap was lower in low-Cd isolines (Harris and Taylor, 2004) and xylem exudation occurred at reduced rate, suggesting that xylem-loading is more active in the high-Cd isoline. Increased retention could also result from increased vacuolar sequestration of Cd within root cells, thus reducing available Cd for root-to-shoot translocation.

1.3.2. Phloem Mobilization of Cadmium into the Grain

Transport of Cd through the phloem can also contribute to Cd accumulation in the grain of durum wheat. Experiments that applied the radioisotope ^{109}Cd to the leaves suggest redistribution of Cd can occur in various wheat genotypes (Cakmak *et al.*, 2000). Harris and Taylor (2004) argued that with reduced shoot Cd pools in low-Cd NIL,

remobilization of Cd into grain was lessened. Greater phloem transport in the high-Cd isoline could also account for (or amplify) differences in Cd levels between the low and high-Cd accumulating NILs (Hart *et al.*, 2005). Re-translocation of Cd from shoot-to-root in low-Cd cultivars during tillering could also account for decreased grain Cd accumulation (Chan and Hale, 2004). Sequestration of Cd in leaf cells may also reduce the available pool of Cd for remobilization, much like in the root system but with a smaller Cd pool.

1.3.3. Interactions Between Cadmium and Zinc for Plant Uptake

Zinc serves a vital role in the metabolism of cells as an enzyme cofactor and structural component (Broadley *et al.*, 2007). Since both Zn and Cd are group IIB transition elements and are predominately present as divalent ions in solution (Chesworth, 1991), the chemical similarity between these ions allows Cd^{2+} to displace Zn^{2+} for adsorption sites in the soil and binding sites of transporters.

Hart and coworkers (2002; 2005) found that Zn competitively inhibits Cd uptake in durum wheat, suggesting a shared uptake mechanism in the root. Moreover, the application of Zn to soil surface during early seedling development may reduce grain Cd accumulation (Hart *et al.*, 2005). Uptake of Cd into durum wheat is elevated in Zn-deficient soils, reiterating that Cd^{2+} can substitute for Zn^{2+} when relatively more abundant (Oliver *et al.*, 1994). Addition of Zn^{2+} to nutrient solutions also decreased Cd in the shoot of spring wheat seedlings (Green *et al.*, 2003). Interestingly, neither accumulation nor partitioning of Zn differed among the root and shoot tissues of low-Cd and high-Cd NILs

(Harris and Taylor, 2004; Hart *et al.*, 2006), suggesting that regulation of grain Cd accumulation does not disrupt the uptake or partitioning of Zn in the plant.

1.3.4. Minor Factors Controlling Accumulation of Cadmium

Other studies found that apoplastic bypass (transport of ions across the endodermial layer in a non-symplastic manner) did not account for Cd accumulation in durum wheat cultivars (Van der Vliet *et al.*, 2007). These authors also indicated that transpiration rate had a minor effect on Cd translocation to the shoot (and most likely grain). Experiments using Indian mustard showed transpiration was linked to Cd uptake (Salt *et al.*, 1995), while not linked for inbred maize lines (Florijn and Van Beusichem, 1993), suggesting transpiration may not be responsible for Cd accumulation in all plants.

1.3.5. Genetic Inheritance of Cadmium Accumulation in Durum Wheat

Studies of inheritance have shown that Cd accumulation is mainly controlled by a single gene, with low-Cd being dominant (Clarke, 1995; Clarke *et al.*, 1997a). This gene resides on chromosome 5B (Knox *et al.*, 2003), and fine-mapping projects are ongoing to pinpoint the exact gene (C.J. Pozniak, University of Saskatchewan, personal communication). Thus, the genetic component that controls accumulation of Cd in grain likely involves a gene product possessing a generic function (i.e. vacuole sequestration), as opposed to one specialized for a xylem or phloem pathway (Harris and Taylor, 2004). Alternatively, the gene may encode a regulatory protein (e.g.: transcription factor) that may control a subset of genes involved in Cd partitioning. Nonetheless, the molecular mechanism regulating Cd accumulation in durum wheat grain remains elusive.

1.4. Possible Mechanisms of Tolerance to Cadmium

Plants have evolved intricate systems to keep essential metals within optimal physiological levels – between deficiency and toxicity – but also to minimize exposure to non-essential metals, like Cd (Clemens, 2001, 2006). Much of our current understanding of metal homeostasis comes from experiments with plants grown under deficient and/or toxic conditions of a given metal, with only a few studies using concentrations typical of agricultural soils. Although analyzing metal homeostasis under normal metal concentrations would be ideal, studies of metal toxicity and tolerance can still be used to infer how mechanisms of metal homeostasis may operate.

Mechanisms of Cd tolerance (Figure 1.1; Clemens, 2006) are comprised of: Uptake, reduced transfer of Cd from the rhizosphere and/or apoplast across the root plasma membrane; Chelation, binding of free Cd^{2+} to buffer/limit interaction with cytosolic enzymes and organelles; Sequestration, removal of Cd (free and complexed) from the cytosol into storage organelles (e.g.: vacuole); Efflux/Distribution, export of Cd into the rhizosphere and/or apoplast or into the xylem or phloem for translocation within the plant. Both chelation and sequestration of Cd can potentially limit cytosolic Cd^{2+} concentrations; however, the broad ion selectivity of transporters involved in uptake and distribution of plant nutrients leads to accumulation of Cd from the soil and throughout the plant, respectively (Clemens *et al.*, 2002).

Some plant species, known as hyperaccumulators, accumulate metals at an order of magnitude greater than non-accumulators, and show increased tolerance towards the specific metals that they accumulate (Chaney *et al.*, 1997; Salt and Krämer, 2000).

Brooks *et al.* (1977) first defined hyperaccumulators as plants that had nickel concentrations greater than 1000 mg kg^{-1} dry weight in their leaves. Cadmium hyperaccumulators are plants with shoot Cd concentrations greater than 100 mg kg^{-1} (Baker and Brooks, 1989). The best-studied Cd-hyperaccumulators are *T. caerulescens* (Baker *et al.*, 1994), *Sedum alfredii* (Yang *et al.*, 2004) and *Arabidopsis halleri* (Bert *et al.*, 2002). Each of these species can also hyperaccumulate Zn, suggesting common pathways for partitioning these metals within the plant. Overall, hyperaccumulators serve as interesting models for investigating how metal tolerance functions in all plants (Clemens *et al.*, 2002).

Several genes involved in the accumulation and tolerance of metals in plants have been identified and characterized in the last two decades, providing insight into the molecular mechanisms of uptake and tolerance. Established eukaryotic systems in yeast have served as tools for pinpointing the function of many plant proteins. Early studies screened Arabidopsis complimentary DNA (cDNA) libraries to identify genes that complemented yeast mutants impaired in metal homeostasis (Eide *et al.*, 1996; Clemens *et al.*, 1998; Grotz *et al.*, 1998). In addition, putative functions of plant genes have been verified by expressing them in yeast strains devoid of a homologous gene to see if the plant gene can restore function. Currently, our understanding of how metal tolerance (and homeostasis) operates is incomplete, and even less is known for economically relevant crops such as wheat, potato, and rice (Clemens *et al.*, 2002).

1.4.1. Cellular Uptake of Cadmium

Plant cells readily accumulate Cd, despite having no known biological function. Cadmium acts as a cofactor for the enzyme carbonic anhydrase under Zn-limited environments in marine diatoms (Lane and Morel, 2000; Lane *et al.*, 2005), suggesting that non-essential metal uptake in plants may stem from ancestral mechanisms for acquiring these metals under micronutrient scarcity. Currently, no Cd-specific uptake transporter has been identified in higher plants; however, studies of the Cd-hyperaccumulator *T. caerulescens* showed high-affinity Cd^{2+} transport that was not inhibited by addition of Zn^{2+} (Lombi *et al.*, 2001). If such a transporter exists, hyperaccumulator plants may also have evolved a unique class of Cd-binding enzymes.

Entry of Cd most likely occurs through transport proteins that mediate the influx of chemically similar divalent cations like Ca^{2+}, Fe^{2+}, and Zn^{2+} (Figure 1.1; Clemens *et al.*, 2002). The ZRT-/IRT-like protein (ZIP) family mediates Fe^{2+} and Zn^{2+} uptake (Guerinot, 2000), and can also transport Cd^{2+} due to a lack of ion selectivity. For instance, AtIRT1 mediates uptake of Fe in root cells of *A. thaliana* (Vert *et al.*, 2002) and was blocked by addition of Zn^{2+}, Mn^{2+}, Co^{2+}, and Cd^{2+} when expressed in yeast (Eide *et al.*, 1996; Korshunova *et al.*, 1999). Expression of *AtIRT1* is induced to facilitate Fe uptake under Fe-limited conditions (Eide *et al.*, 1996), and both Fe and Cd root levels increase (Connolly *et al.*, 2002). Root influx of Cd^{2+} and root Cd accumulation also increased in pea seedlings under Fe deficiency (Cohen *et al.*, 1998), which has been attributed to the induction of a gene homologous to *AtIRT1*. Therefore, up-regulation of the Fe uptake system can lead to Cd accumulation in numerous plant species. Other ZIP transporters involved in Zn homeostasis may also contribute to Cd uptake. AtZIP1,

AtZIP2, and AtZIP3 conferred Zn^{2+} uptake activities when expressed in *S. cerevisiae*, but this activity was hindered in the presence of Cd^{2+} (Grotz *et al.*, 1998).

Uptake of Cd^{2+} has also been observed through Ca^{2+} channels. The low-affinity cation transporter (LCT1) from *T. aestivum* is a Ca^{2+} channel that is also permeable to Cd^{2+} (Clemens *et al.*, 1998). Yeast expressing *LCT1* showed increased sensitivity to Cd when compared to control strains. Experiments measuring stomatal behaviour in *A. thaliana* found Cd^{2+} interfered with the function of Ca^{2+} channels in guard cells and addition of Ca^{2+}-channel inhibitors suppressed the inhibitory effect of Cd^{2+} (Perfus-Barbeoch *et al.*, 2002). Moreover, these channels were permeable to Cd^{2+} and may serve as a route of entry into the cytosol (Figure 1.1).

Total Cd accumulation does not differ between low-Cd and high-Cd accumulating durum wheat NILs, suggesting that the isolines have equivalent uptake capacities (Harris and Taylor, 2004). Differences in net Cd^{2+} influx at the root surface was negligible between isolines (Harris and Taylor, 2004) and also between low-Cd and high Cd-accumulator cultivars (Farrell *et al.*, 2005). Accumulation of Cd was reduced with the application of Zn^{2+} (Hart *et al.*, 2002; Hart *et al.*, 2005), suggesting that the route of Cd^{2+} may be through a Zn uptake transporter, such as one homologous to the Arabidopsis ZIP family.

1.4.2. Chelation of Cytoplasmic Cadmium

Most metal ions are bound by ligands within the cell, and are not present as hydrated free ions. Chelator ligands can buffer cytosolic concentrations of essential metals, and reduce unwanted interactions between non-essential metals and enzymes

(Clemens, 2006). For instance, Cd^{2+} can interfere with protein function by binding to nitrogen and sulfur donors of amino acid residues.

Phytochelatins (PCs) bind to a wide array of metals (Maitani *et al.*, 1996; Schmöger *et al.*, 2000). Zenk (1996) reported the binding affinity of PCs in plant suspension cells to be Cd > Pb > Zn > gold (Ag) > Hg > As > Cu. Phytochelatins have the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$, where *n* varies from 2–11 repeats. These peptides are not synthesized by translation of mRNA, but rather in a transpeptidase reaction catalyzed by phytochelatin synthase (PCS; Grill *et al.*, 1989). This enzyme transfers the dipeptide moiety of GSH (γ-Glu-Cys-Gly) to another GSH or a pre-existing PC oligomer to form the long peptide chain of PCs. Impairments in the pathway of GSH synthesis can result in PC-deficient and Cd-sensitive plants, such as γ-glutamylcysteine synthetase mutants in Arabidopsis (May and Leaver, 1994; Howden *et al.*, 1995a; Cobbett *et al.*, 1998). Individual GSH compounds can also bind Cd (Figure 1.1). In yeast, which are devoid of PC synthesis, GS_2-Cd complexes are formed in the cytosol and are sequestered in the vacuole (Li *et al.*, 1997b).

Cadmium (Cd) is the most prominent activator of *PCS*, perhaps indicating the relevance of PCs in tolerance to Cd (Clemens *et al.*, 1999). In fact, the first plant *PCS* genes were identified from Arabidopsis (*AtPCS1*) and wheat (*TaPCS1*) by screening cDNA libraries for enhanced Cd tolerance in *S. cerevisiae* (Clemens *et al.*, 1999; Vatamaniuk *et al.*, 1999). Furthermore, *atpcs1* mutants are devoid of detectable PCs and are Cd-hypersensitive, reiterating the importance of PCs in Cd tolerance *in planta* (Howden *et al.*, 1995b; Ha *et al.*, 1999).

Phytochelatins are known to be a universal response to acute Cd toxicity (Sanità di Toppi and Gabbrielli, 1999), but whether they are prominent at Cd concentrations typical of agricultural soils (e.g.: nM range) remains to be shown. Wagner (1993) suggested that PCs are only a major player in soils with high Cd levels, while other complexes of GSH and organic acids predominate at lower levels. Marentes and Rauser (2007) found PCs bound 79% of root Cd at 500 nM Cd in durum wheat; however, when the concentration was lowered to 50 nM Cd, PC-Cd complexes only bound 37% of root Cd. These authors found no detectable trace of free Cd^{2+}, suggesting that other molecules form stable complexes with Cd in the cytoplasm (e.g.: GSH). Other studies have shown that PC contents of low-Cd and high-Cd accumulating durum wheat NILs were equivalent (Hart *et al.*, 2006), suggesting PCs play a minor role in regulating Cd accumulation when grown on agricultural soils.

Other ligands that could contribute to chelation of Cd in the cytoplasm include metallothioneins and organic anions. Metallothioneins are gene-encoded, cysteine-rich peptides that have only recently been isolated and characterized in plants (Cobbett and Goldsbrough, 2002). Their involvement in the chelation and detoxification of Cd is not well understood, but theoretically the thiol groups of these peptides could bind Cd. Recently, a metallothionein isolated from durum wheat increased Cd tolerance in *Escherichia coli*; however, structural models indicated the lack of a metal binding site (Bilecen *et al.*, 2005). These authors suggested the association of Cd only occurred due to interactions with the cysteine thiol groups, thus the *in vivo* function of the metallothionein is not metal detoxification. Chelation of Cd^{2+} by citrate and malate may also occur in the cytoplasm (Wagner, 1993).

1.4.3. Sequestration of Cadmium in Cellular Organelles

Removal of non-essential metals from the cytoplasm into storage organelles ensures spatial separation from the cytosol. Transfer of metal-conjugate complexes into the vacuole is mediated by an array of transporters localized at the tonoplast. Numerous loss-of-function studies in yeast indicate these proteins are key components of metal detoxification, as mutants have reduced sequestration activity and become hypersensitive to the metal that is transported (Ortiz *et al.*, 1992; Szczypka *et al.*, 1994; Ortiz *et al.*, 1995).

A variety of studies have suggested that storage of PC-Cd complexes in the vacuole occurs in plants. Mesophyll cells from tobacco contain nearly all Cd and PCs in the vacuole (Vögeli-Lange and Wagner, 1990), and isolated oat tonoplast vesicles show ATP-dependent, orthovandate-sensitive uptake of PC-Cd complexes (Salt and Rauser, 1995). Phytochelatin complexes are sequestered into the vacuole in *Schizosaccharomyces pombe* by the tonoplast-localized, half-size ATP-binding cassette (ABC) transporter heavy metal tolerance factor 1 (HMT1) (Ortiz *et al.*, 1992; Ortiz *et al.*, 1995). These results suggest that an ABC transporter is a likely candidate to sequester PC-Cd (Figure 1.1). Recently, a HMT1 homolog has been identified in the nematode *Caenorhabditis elegans* and *cehmt1* mutants are hypersensitive to Cd (Vatamaniuk *et al.*, 2005). However, an inventory of Arabidopsis ABC transporters found no HMT1-like transporter (Sánchez-Fernández *et al.*, 2001), nor has any other study identified a plant transporter with this function.

Similar to PC-Cd complexes, transfer of GSH-Cd conjugates across the tonoplast is not well understood in plants. Sequestration of GS_2-Cd in the vacuole of *S. cerevisiae* is mediated by the full-size ABC transporter yeast cadmium factor 1 (YCF1) (Li *et al.*, 1996b; Li *et al.*, 1997b), and yeasts devoid of this activity are hypersensitive to Cd (Szczypka *et al.*, 1994). Heterologous expression of *AtMRP3/AtABCC3* in *ycf1* mutants rescues Cd tolerance, suggesting AtABCC3 may serve as a glutathione conjugate pump in Arabidopsis (Figure 1.1; Tommasini *et al.*, 1998). However, evidence demonstrating that GS_2-Cd can act as a substrate is lacking, and there are no reports of the *atabcc3* mutant in the literature.

The cation-exchange transporter (CAX) family of Cd^{2+}/H^+ antiporters has also been suggested to transport Cd into the vacuole (Figure 1.1; Salt and Wagner, 1993). Overexpressing *AtCAX2* in tobacco resulted in 3-fold higher Cd content in the root, and higher accumulation of Cd than Ca and manganese (Mn) (Hirschi *et al.*, 2000). Analysis of the transport kinetics of tonoplast vesicles isolated from AtCAX4-overexpressing tobacco plants demonstrated increased Cd accumulation, with an ion selectivity of $Cd^{2+} > Zn^{2+} >> Ca^{2+} >> Mn^{2+}$ (Koren'kov *et al.*, 2007). These results suggest free Cd^{2+} is not only actively transported into the vacuole, but is also available in the cytosol. However, the binding affinities of Cd to the thiol residues of GSH and PCs suggests that most of intracellular Cd is chelated (Vatamaniuk *et al.*, 2000). Therefore, how CAXs function *in planta* might differ from the isolated tonoplast vesicles.

1.4.4. Efflux/Distribution Systems for Cadmium

In addition to chelation and sequestration mechanisms that can accumulate metals within the cell, efflux pumps may also affect to total plant accumulation. Such transporters exist in single-celled organisms, and serve to remove excess metals from the cytosol into the environment. Such simplistic models are not realistic in plants. Rather, plasma membrane-localized transporters can release metals into the rhizosphere and/or transfer metals into the xylem for distribution within the plant, especially to regions that are deficient for a particular micronutrient. Consequently, non-essential metals can also use these efflux systems, leading to accumulation within the shoot tissue.

Xylem loading of Cd may be controlled by heavy metal transporting P-type ATPases (HMAs) (Axelsen and Palmgren, 2001). Both AtHMA2 and AtHMA4 are involved in translocation of Zn, as both are expressed in the root vascular tissue, are plasma membrane-localized, and *athma2 athma4* double mutants have Zn-deficient phenotypes (Hussain *et al.*, 2004). *AtHMA4* can also complement the Cd-hypersensitive *ycf1* yeast mutant (Mills *et al.*, 2005), and overexpression of AtHMA4 in Arabidopsis increases Cd levels in shoots (Verret *et al.*, 2004), suggesting these proteins can also substitute Cd^{2+} for Zn^{2+} (Figure 1.1). Other factors affecting xylem loading include the availability of metals in the cytosol, and the number of transporters located on the plasma membrane (Clemens, 2006).

Cadmium may also be transported in the xylem as a PC-Cd complex (Figure 1.1), as these complexes were detected in the shoot tissue of PC-deficient Arabidopsis mutants expressing *TaPCS1* under a root-specific promoter (Gong *et al.*, 2003). Conversely, other studies suggest that PC-Cd complexes are not transported in the xylem since there is no

direct evidence of these compounds in the xylem sap (Pomponi *et al.*, 2006). Evidence for transport of histidine-nickel complexes in the xylem has been reported for Ni-hyperaccumulators of *Alyssum* species, which activate histidine synthesis under elevated nickel levels (Krämer *et al.*, 1996).

Recently, Kim *et al.* (2007) reported that the ABC transporter AtPDR8 from *A. thaliana* functions as a Cd efflux pump and may play a role in Cd resistance (Figure 1.1). Expression of *AtPDR8* was induced under Cd exposure, and AtPDR8-overexpressing and AtPDR8-knockdown plants had lower and higher Cd content than wild-type, respectively. AtPDR8 is localized to the plasma membrane of root hair and epidermal cells, and transport assays using isolated protoplasts from AtPDR8-overexpressing plants demonstrated increased Cd^{2+} efflux when compared to wild-type (Kim *et al.*, 2007). *AtPDR8* expression was also elevated under Pb stress, and AtPDR8-overexpressors were more resistant to Pb^{2+}, suggesting AtPDR8 may confer resistance to a range of metals.

In summary, researchers are only starting to understand what controls Cd uptake, accumulation and partitioning in the plant. Unanswered questions and sometimes-contradictory results indicate that these systems still have missing pieces. Much of our knowledge has come from the model plant Arabidopsis, and little is known about the molecular mechanisms of metal homeostasis in agricultural crops, or if similar components are involved. For durum wheat, identifying the components involved in metal uptake and partitioning will allow researchers and breeders to target specific genes that might reduce Cd levels in the grain, while ensuring that essential metals, like Zn, are still available to support crop yield.

1.5. Microarray Analysis of Durum Wheat Isolines that Differ in Accumulation of Cadmium in Grain

Microarrays offer a high-throughput approach to investigating differences in gene expression in plants (Galbraith, 2006). Microarray studies with wheat have provided insight into several intricate processes including grain hardness (Clarke and Rahman, 2005), leaf senescence (Gregersen and Holm, 2007), nitrogen use (Ruuska *et al.*, 2008), and response to environmental stresses such as cold tolerance (Gulick *et al.*, 2005), salinity (Kawaura *et al.*, 2006; Kawaura *et al.*, 2008), and drought (Mohammadi *et al.*, 2007; Mohammadi *et al.*, 2008). A comparative microarray approach between *A. thaliana* and the Cd-hyperaccumulator *A. halleri* identified a core set of genes responsible for Cd tolerance, including a putative ZIP transporter and nictotinamide synthase (Weber *et al.*, 2004).

To identify genes potentially involved in Cd accumulation in grain of durum wheat, microarray analysis was performed on root samples of low-Cd and high-Cd accumulating NILs (Appendix A; N.S. Harris and G.J. Taylor, unpublished). The goal of this experiment was to compare genotypes (isolines) grown under constant environmental conditions. In contrast to numerous studies that exposed plants to Cd concentrations that elicit a toxic response, durum wheat seedlings were grown under non-phytotoxic concentrations ($[Cd^{2+}]$ = 14.4 pM) to resemble typical agricultural soil conditions. Statistical analysis identified less than 40 genes as being differentially expressed between the two genotypes, including genes encoding proteins for glycosyl hydrolases, ß-glucosidases, germins, and omega-3 fatty acid desaturases. One of the candidate genes is a putative, multidrug resistant-associated protein (MRP) transporter that showed higher

expression in the low-Cd isoline. The putative amino acid sequence encoded by this gene showed highest sequence similarity to both *O. sativa* and *A. thaliana* MRP3 proteins.

1.6. ATP-Binding Cassette Subfamily C

ABC transporters are one of the largest protein families, encompassing over 3000 members that span all three kingdoms (Holland *et al.*, 2003). Although over 120 ABC transporters have been identified in the Arabidopsis genome (Sánchez-Fernández *et al.*, 2001), only a small portion have been characterized (Rea, 2007). Already ABCs are known to mediate transport of a wide variety of substrates involved in lipid catabolism, antibiotic resistance, alkaloid import, auxin transport, stomatal regulation, vacuolar xenobiotic sequestration, assembly of redox proteins, and metal tolerance.

The MRP transporters represent the C subfamily of the ABC transporter family in plants. Although MRPs were originally named from the human MRP1 overexpression in drug-resistant cancer cells (Cole *et al.*, 1992), the function in plant extends beyond 'multi-drug' involvement. Thus, a unified nomenclature has been proposed for plant ABC transporters to provide clarity within the field (Verrier *et al.*, 2008). Plant MRPs will be designated as ABCCs throughout this thesis.

Proteins in the ABCC subfamily are known for their MgATP-dependent transport of glutathione S-conjugates (GS-X) of metals and organic anions across membranes, albeit transport of unconjugated compounds has also been observed (Lu *et al.*, 1998). All ABC transporters have an arrangement of alternating hydrophobic transmembrane domains (TMD) and nucleotide-binding folds (NBF) (Garcia *et al.*, 2004; Klein *et al.*, 2006). ABCC proteins from eukaryotes exist as full-size transporters and have a

conserved organization of $(TMD\text{-}NBF)_2$ (Garcia *et al.*, 2004). The ABCC subfamily is defined by a conserved and highly hydrophobic N-terminal extension (NTE) (Tusnády *et al.*, 1997). The function of the NTEs in plants is poorly understood, but studies of the human ABCC1 and the yeast YCF1 suggest the NTE is vital for protein trafficking (Mason and Michaelis, 2002; Westlake *et al.*, 2005).

1.6.1. ABCCs in *Saccharomyces cerevisiae*

Yeast has six members in the ABCC subfamily of ABC transporters (Decottignies and Goffeau, 1997; Taglicht and Michaelis, 1998). The major vacuolar GS-X pump YCF1 confers Cd tolerance by transporting bis(glutathionato)cadmium into the vacuole (Li *et al.*, 1996b; Li *et al.*, 1997b). YCF1 has also been linked to vacuolar sequestration of As (Ghosh *et al.*, 1999) and Hg (Gueldry *et al.*, 2003), suggesting a versatile role in metal detoxification. A close homologue of YCF1, bile pigment transporter 1 (BPT1), localizes to the vacuolar membrane, mediates GS-X transport of bile acids, and plays a minor role in Cd detoxification (Petrovic *et al.*, 2000; Klein *et al.*, 2002; Sharma *et al.*, 2002). Furthermore, *ycf1 bpt1* double mutants have greater Cd sensitivity than the *ycf1* mutant alone (Sharma *et al.*, 2002), and cells are devoid of any detectable GS-X transport activity (Klein *et al.*, 2002).

1.6.2. ABCCs in Higher Plants

There are 15 ABCCs in *A. thaliana* (Kolukisaoglu *et al.*, 2002) and 17 ABCCs in *O. sativa* (Klein *et al.*, 2006). Comparison of homologous genes between these plants showed a conserved gene organization for the ABCC subfamily, suggesting a common

ancestor prior to monocot and dicot divergence (Jasinski *et al.*, 2003; Garcia *et al.*, 2004). Even with a comprehensive inventory of rice ABCCs annotated from the completed genome sequence (International Rice Genome Sequencing Project, 2005), no functional studies exist in the literature for this species.

Only five ABCCs (AtABCC1 through 5) have been functionally characterized in Arabidopsis (Klein *et al.*, 2006), although *AtABCC6* and *AtABCC7* have also been shown to be induced under Cd toxicity (Gaillard *et al.*, 2008). Substrate analysis shows AtABCC1, 2, 3, and 5 mediate the transport of the model glutathione complex *S*-(2,4-dinitrophenyl)glutathione, and AtABCC1 and 2 can transport oxidized glutathione (Lu *et al.*, 1997; Lu *et al.*, 1998; Tommasini *et al.*, 1998; Klein *et al.*, 2003). Only *AtABCC3* and *4* confer Cd tolerance when heterologously expressed in the Cd-hypersensitive *ycf1* mutant (Tommasini *et al.*, 1998; Klein *et al.*, 2006). In addition, expression of *AtABCC3* is strongly induced when exposed to 50 μM Cd_2Cl, indicating AtABCC3 could play an important role in Cd detoxification (Bovet *et al.*, 2003; Bovet *et al.*, 2005). AtABCC1 and 4 localize to the tonoplast and plasma membrane, respectively (Geisler *et al.*, 2004; Klein *et al.*, 2004). Klein *et al.* (2006) suggested AtABCC4 functions as an efflux pump for Cd detoxification in Arabidopsis on the basis that the transporter localizes to the plasma membrane.

In the absence of a sequenced genome, information regarding ABCCs for crop species other than rice is minimal. Four *Zea mays* (maize) ABCCs have been identified, including the ZmABCC3, which is involved in the anthocyanin biosynthesis pathway (Goodman *et al.*, 2004) and *ZmABCC1*, which was induced under exposure to the herbicide aminotriazole (Swarbreck *et al.*, 2003). *ZmABCC3* is expressed in the shoot,

leaf, husk, and tassel, while *ZmABCC1* and *ZmABCC2* are expressed in seedlings and adult leaf. These expressional differences suggest developmental and tissue-specific functions for ABCCs; however, analysis of substrate specificities and transport kinetics were not reported.

An understanding of the function of ABCCs in wheat is even more incomplete. Of the two cDNAs from *T. aestivum* shown to encode putative transporters, *TaABCC1* (partial cDNA) and *TaABCC2* (full-length cDNA), only *TaABCC1* showed increased transcript levels upon xenobiotic exposure (Theodoulou *et al.*, 2003).

1.7. Research Objectives

ABCCs appear to be involved in the detoxification of Cd in yeast (YCF1) and Arabidopsis (AtABCC3) by transporting glutathione conjugates across the tonoplast. Retention of Cd in root cells of durum wheat by such a transporter could potentially explain the physiological regulation of grain Cd accumulation, as reduced Cd availability for root-to-shoot translocation would lead to lower Cd content in the grain. Sequestration of Cd in leaf cells could also explain decreased Cd transport in the phloem of the low-Cd isoline. Microarray analysis of root tissue from low-Cd and high-Cd NILs identified a gene encoding a putative ABCC protein that was more highly expressed in the low-Cd isoline. I defined this gene as *T. turgidum ABCC3* (*TtABCC3*) because of its sequence homology to *AtABCC3* and *OsABCC3*.

In order to confirm that *TtABCC3* encodes a full-length ABCC transporter, and to test the hypothesis that TtABCC3 is involved in Cd sequestration, my research addressed the following objectives:

1. Isolation and sequencing of the full-length coding region of *TtABCC3*.
2. Analysis of the *TtABCC3* sequence and comparison to other plant ABCCs.
3. Expression of *TtABCC3* in the Cd-hypersensitive *ycf1* mutant to test if Cd tolerance is restored.

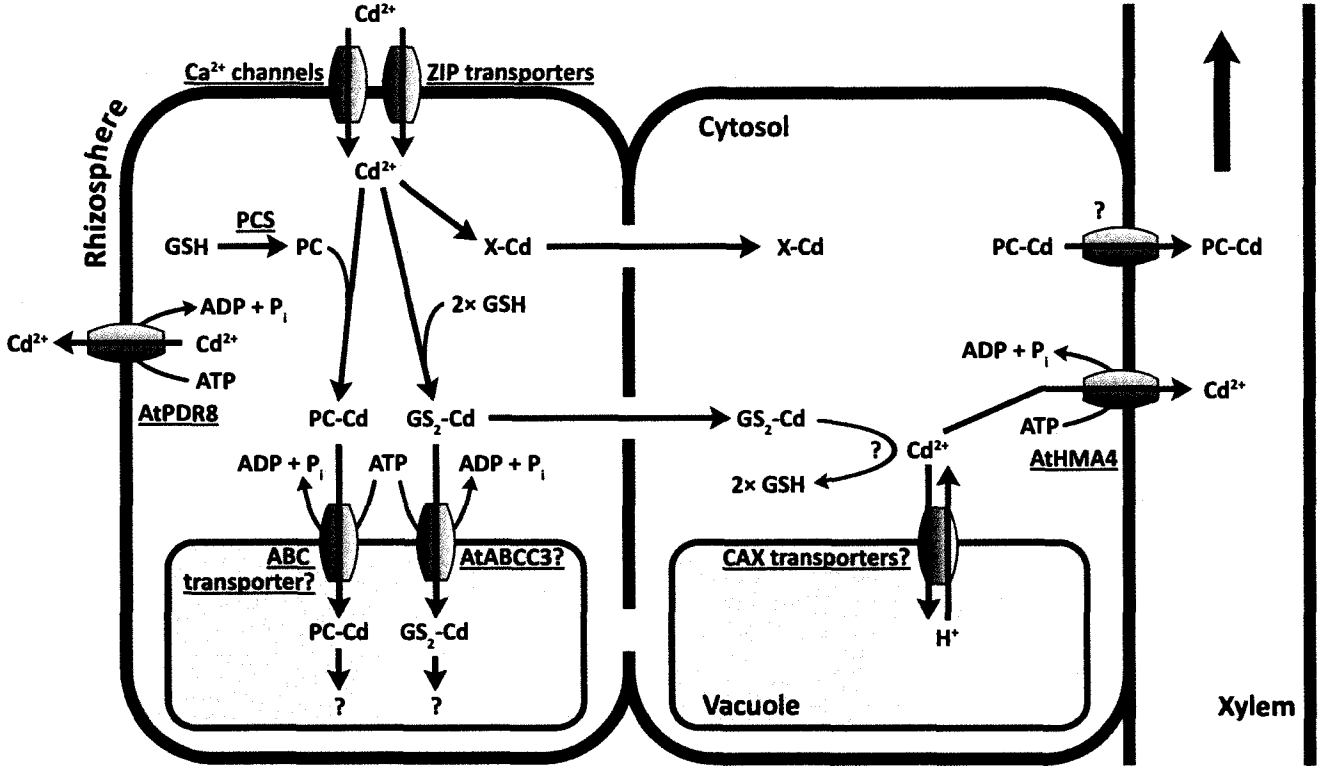

Figure 1.1. Possible mechanisms of plant tolerance to cadmium.
Cellular uptake of Cd^{2+} from the rhizosphere and/or apoplast likely occurs through Ca^{2+} channels and Fe^{3+}/Zn^{2+} ZIP transporters (top left). Cadmium ions are bound by phytochelations (PCs), glutathione (GSH) or other ligands (X) once in the cytosol. PCs are enzymatically generated from GSH by phytochelatin synthase (PCS). Cd-complexes can move between cells via the plasmodesmata. Sequestration of PC-Cd complexes in the vacuole is predicted to occur by an ABC transporter homologous to HMT1 (yet to be identified). Rescue of the Cd-hypersensitive, tonoplast-localized *ycf1* mutant by *AtABCC3* expression suggests this protein may transport glutathione conjugates into the vacuole. Fates of PC-Cd and GS_2-Cd in the vacuole are unknown. The CAX Cd^{2+}/H^+ anti-port transporters may also facilitate transfer of Cd^{2+} into the vacuole. Translocation of Cd to aerial tissues is dependent on xylem loading mediated by transporters located in the plasma membrane of xylem parenchyma cells. AtHMA4 mediates the transport of Cd^{2+}, and PC-Cd complexes may also be transferred into the xylem by an unknown protein. Plasma membrane-localized AtPDR8 (middle left) is expressed in the root epidermis and exports Cd^{2+} into the rhizosphere as a resistance mechanism. See text for full explanations. Adapted from Clemens (2006).

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2. Isolation of *TtABCC3* from durum wheat (*Triticum turgidum* L. var. *durum*)

2.1. Introduction

In order to assess the hypothesis that TtABCC3 is involved in the vacuolar sequestration of cadmium (Cd), the full-length coding region must be isolated and sequenced.

Wheat has one of the largest genomes in the plant kingdom. Bread wheat (*T. aestivum*) is comprised of three ancestral genomes (A, B, and D), each with 7 chromosomes. At over 16,000 Mb (Arumuganathan and Earle, 1991), the genome is 40-fold larger than rice (International Rice Genome Sequencing Project, 2005) and 125-fold larger than Arabidopsis (The Arabidopsis Genome Initiative, 2000). Durum wheat is an evolutionary progenitor of bread wheat, and has a smaller genome containing only the A and B genomes. Whole genome sequencing (for both wheat species) is not economically viable because of high sequencing costs associated with the genome size and the current technology of shotgun sequencing (Stein, 2007). Partial sequencing of the wheat genome by The International Wheat Genome Sequencing Consortium (http://www.wheatgenome.org) is currently underway, including the D-genome donor species *Aegilops tauschii* (http://wheat.pw.usda.gov/PhysicalMapping) and a pilot project on chromosome 3B (Moolhuijzen *et al.*, 2007). Along with the requirement for several-fold coverage of the genome, the sequencing process is further complicated by polyploidy (genetic redundancy) and the presence of repetitive DNA dispersed throughout the genome. Smith and Flavell (1975) estimated wheat genomes contain 80%

repetitive sequences. Thus, clusters of disconnected genes are created when compiling genome sequences as gaps remain from highly similar intergenic regions.

In the absence of a sequenced genome, scientists rely on expressed sequence tags (ESTs) to obtain gene sequence information. ESTs are short (200-800 bp), single-pass sequence reads of complimentary DNA (cDNA) libraries that are usually random and redundant (Nagaraj *et al.*, 2007). For species with large genomes like wheat, these EST-based datasets offer an alternative to whole genome sequencing, and are often termed the 'poor-man's genome' (Rudd, 2003). Wheat EST databases are estimated to have 60% gene coverage (Gill *et al.*, 2004). Thus, ESTs do not represent all the genes in the wheat genome, especially for transcripts that are rare or tissue-specific. However, ESTs provide a foundation for gene discovery and transcriptome-based experiments.

The primary source for raw EST sequence data is the National Center for Biotechnology Information (NCBI) dbEST (Boguski *et al.*, 1993), which currently exceeds 1 million *Triticum* ESTs. Raw ESTs are not highly informative, requiring processing using a variety of computational tools to cluster and assemble them for database searching and functional annotation (Nagaraj *et al.*, 2007). Several EST-based assembly databases exist for wheat including the Dana-Farber Cancer Institute (DFCI) Gene Index Project (http://compbio.dfci.harvard.edu/tgi)[1], The Institute for Genome Research (TIGR) Plant Transcript Assemblies (http://www.tigr.org/tdb/tgi; Childs *et al.*, 2007), Plant Genome Database (http://www.plantgdb.org/; Dong *et al.*, 2004), and NCBI UniGene (http://www.ncbi.nlm.nih.gov/UniGene; Pontius *et al.*, 2003). The DFCI database uses stringent assembly parameters to generate high-quality, unique contigs by

[1] The Gene Index Project was originally a TIGR resource, but switched to DFCI in 2006.

combining ESTs into longer, virtual transcripts called tentative consensuses (TCs). UniGene represents the least powerful resource as no assembly sequence is provided, just a list of the clustered ESTs. TIGR contains the most current information on EST assemblies, processing over 860,000 ESTs into approximately 65,000 assemblies and 270,000 singletons to represent 335,000 unique gene sequences (or roughly 117,000 genes per homeologous genome). Given that the rice genome has an estimated 40,000 genes (International Rice Genome Sequencing Project, 2005), these assemblies likely include the core set of genes in the wheat genome (Stein, 2007), but several assemblies may be redundant and the large proportion of singleton ESTs suggests incomplete gene coverage.

Even with numerous EST resources, the TC or singleton sequences are often 3'-end biased and result in partial sequence coverage for genes with long coding regions (Nagaraj *et al.*, 2007). Therefore, other strategies are required to obtain full-length sequences, such as screening of cDNA libraries, homology-based primer design, and/or rapid amplification of cDNA ends (RACE). Within the latter technique, several variants have been developed including ligation-anchored PCR (LA-PCR; Troutt *et al.*, 1992) and rolling circle amplification-RACE (RCA-RACE; Polidoros *et al.*, 2006). In comparison to other RACE techniques, RCA-RACE (Figure 2.1) has the advantages of multiple amplification steps (enriching low abundant transcripts), minimal optimization, and no first-strand cDNA modifications, such as A-tailing or adaptor ligation. Furthermore, the intramolecular circularization of each cDNA is more efficient than the attachment of an anchor sequence (Schaefer, 1995), ultimately leading to a larger pool of useable template for downstream applications.

Members of the ATP-binding cassette subfamily C (ABCC) are prone to incomplete sequence coverage in EST assembly databases since these genes encode proteins of approximately 1,500 amino acids. The protein structure of ABCC transporters has several conserved features. Almost all eukaryotic ABCCs contain two transmembrane domains (TMDs) and two cytosolic nucleotide-binding folds (NBFs), which are arranged TMD-NBF-TMD-NBF (Holland *et al.*, 2003). These domains are preceded by the N-terminal extension (NTE) domain, which is only found within the ABCC subclass. Within each NBF the Walker A and B motifs (Walker *et al.*, 1982) flank the ABC signature sequence (Higgins *et al.*, 1986; Hyde *et al.*, 1990) and despite sequence variation within each motif, the order of the motifs is always maintained (Rea, 2007).

Here I describe the isolation of *TtABCC3* from durum wheat, a putative glutathione-conjugate transporter identified by microarray analysis to be differentially expressed between near-isogenic lines (NILs) that differ in grain Cd accumulation, with *TtABCC3* showing higher expression in the low-Cd isoline (Appendix A; N.S. Harris and G.J. Taylor, unpublished). Given that other ABCCs, such as the yeast cadmium factor 1 (YCF1) (Tommasini *et al.*, 1998) and Arabidopsis AtABCC3 (Li *et al.*, 1996), are involved in metal detoxification, vacuolar sequestration of Cd in the root by TtABCC3 could limit root-to-shoot translocation. The full-length coding sequence of *TtABCC3* was acquired by combining the resources of ESTs datasets and the novel method of RCA-RACE. Sequencing of *TtABCC3* resulted in two non-homeologous genes encoding putative ABCCs that correspond to the A and B genome.

2.2. Materials and Methods

2.2.1. Plant Growth and Analysis

Two pairs of durum wheat NILs that differ in grain Cd accumulation (Low/High), 8982-TL-L/H and 8982-SF-L/H (Clarke *et al.*, 1997), were used in these experiments. Field studies have shown that mature grain from the high-Cd lines contains 2.5-fold greater concentrations of Cd than the low-Cd lines (Clarke *et al.*, 2002). Seeds were surface sterilized in 1.2% NaOCl for 20 min, rinsed in reverse osmosis (RO) water (<3 $\mu S\ cm^{-1}$), and imbibed for 36 h in an aerated solution of 1 mM $CaCl_2$ and 5 mg L^{-1} Vitavax fungicide (Uniroyal Chemical Ltd, Calgary, AB, Canada). Germinated seeds were placed on nylon mesh suspended over 10 L of aerated chelator-buffered nutrient solution. This nutrient solution was prepared in RO water and contained 1.0 mM $Ca(NO_3)_2$, 0.3 mM $Mg(NO_3)_2$, 0.3 mM NH_4NO_3, 0.25 mM KNO_3, 0.1 mM K_2HPO_4, 0.1 mM K_2SO_4, 50 μM KCl, 100 μM $Fe(NO_3)_3$, 10 μM H_3BO_3, 0.2 μM Na_2MoO_4, 10 μM $ZnSO_4$, 2 μM $CuSO_4$, 1 μM $MnSO_4$, 0.5 μM $CdCl_2$, 0.1 μM $NiCl_2$, 138.6 μM N-(2-hydroxyethyl)ethylenediaminetriacetic acid (HEDTA), 1.42 mM KOH, and 2 mM MES [2-(N-morpholino)ethanesulfonic acid] buffer (pH 6.0). HEDTA was provided in a 25 μM excess over the total concentration of transition metal cations to chelate Cd and micronutrient metals in solution, thereby buffering the free metal activities at environmentally relevant levels (Parker *et al.*, 1995b; Parker and Norvell, 1999). GEOCHEM-PC (Parker *et al.*, 1995a) was used to calculate free ion/ligand/buffer activities (Table 2.1). The free activity of Cd^{2+} was 14.4 pM.

Seedlings were grown for 3 d in the dark, and then a further 4 d in a controlled environment growth chamber with day/night temperatures of 21/16°C (16/8 h), and a

photon flux density of 450 μmol m^{-2} s^{-1}. The caryopses were removed after 7 d and seedlings were transferred to 10 L polyethylene buckets (under the same growth conditions) containing aerated, chelator-buffered nutrient solution as described above. Each bucket held two seedlings, supported independently by a polyethylene mesh basket mounted in opaque polycarbonate lids. Twelve buckets were prepared for each TL-L/H and SF-L/H isoline, totalling 48 buckets with 96 seedlings. All buckets were suspended in a common water bath to maintain a consistent root temperature and limit temperature fluctuations between buckets. Throughout the growth period, solution pH and electrical conductivity (EC) were monitored daily, and RO water was added to maintain constant solution volume. If pH deviated from 6.0±0.1, solutions were adjusted with 1.25 N HNO_3 or KOH. Electrial conductivity was used to estimate nutrient solution depletion. Equal volumes of two daily addition stock solutions (Stock 1: 40 mM KH_2PO_4, 210 mM NH_4NO_3, 420 mM KNO_4, 20 mM $(NH_4)_2SO_4$; Stock 2: 20 mM $Ca(NO_3)_2$, 40 mM $Mg(NO_3)_2$, 0.4 mM H_3BO_3) were added to adjust the solution EC to the nominal level (600 μS cm^{-1}). The composition of the daily addition solution was adapted from Chaney *et al.* (1992) and was optimised in preliminary experiments to maintain shoot nutritional status and to minimize solution pH fluctuation.

Hydroponic and field experiments have consistently demonstrated that seedlings express the low/high Cd accumulation phenotype, and that Cd concentration in seedling shoots is a reliable indicator of the Cd content in the mature grain (McLaughlin *et al.*, 1999; Archambault *et al.*, 2001). Therefore, mature plants were not required to differentiate between low/high NILs and plants were harvested 14 d after being transferred to buckets (21-d-old plants). The roots were triple rinsed in RO water and the

seedlings were divided into shoot and root tissues, blotted dry, flash-frozen and immediately stored at -80°C. The root and shoot tissues for both seedlings within each bucket were combined.

For analysis of Cd in shoots, frozen tissue was finely ground with chilled mortars and pestles, and sub-samples (1 g) were oven-dried at 65°C overnight. Samples were digested in a 5 mL : 2 mL mixture of trace-metal grade concentrated HNO_3 and 30% H_2O_2, diluted to 50 mL with deionized water (>18 MΩ purity) and Cd concentrations were determined by graphite furnace atomic absorption spectroscopy (GF-AAS) on an AAnalyst 700 (PerkinElmer, Waltham, MA, USA). A National Institute of Standards and Technology (NIST) reference material (NIST no. 8436, durum wheat flour) was included in each batch for quality control. Recovery of the certified Cd concentration (0.11±0.05) was 94±9% (mean±SD). Student's *t*-test was used to test for segregation of shoot Cd accumulation within each isoline pair. Ground root tissue (0.5 g) was randomly pooled into groups of 2-3 seedling pairs (4-6 seedlings) after excluding low-Cd or high-Cd samples that were above the upper limit or below the lower limit of the 99% confidence interval for shoot Cd concentration, respectively. Total RNA was extracted from each of the four TL-L/H and SF-L/H groupings (16 pools total) for microarray analysis (see Appendix A).

2.2.2. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from 50 mg of frozen root tissue from all 16 pools using the RNeasy Mini kit (QIAGEN, Mississauga, ON, Canada) and on-column DNase I treatment. Samples were quantified on a ND-1000 spectrophotometer (NanoDrop,

Wilmington, DE, USA). qRT-PCR reactions were carried out using the QuantiTech SYBR Green RT-PCR kit (QIAGEN) using 200 ng total RNA, and 1 μM of each primer (Table 2.2). Cycling conditions were 50°C/20m, 95°C/15m, 40×(94°C/15s, 57°C/30s, 72°C/30s), and included melting curve analysis (60–95°C) to assess primer-dimer formation. Reactions were measured on a 7500 Fast Real-Time PCR System and analyzed by SDS v1.4 software (Applied Biosystems, Foster City, CA, USA), with a manual threshold level of 1000. All samples were measured in triplicate. RNA levels were normalized against the endogenous reference gene *β-Actin* (GenBank no. AB181991; Table 2.2), and relative quantifications between low-Cd and high-Cd NILs were determined using the standard curve method. Standards were based on serial dilutions of each amplicon (*TtABCC3* 200 bp; *β-Actin* 184 bp) that were previously cloned into pGEM-T (Promega, Madison, WI, USA) to prevent cDNA degradation.

2.2.3. *TtABCC3* Contig Construction

Assembled EST sequences for the *TtABCC3* contig were obtained by searching the DFCI *T. aestivum* gene index release 10.0 (http://compbio.dfci.harvard.edu/tgi) using the microarray 70-mer probes with the unique identification number (UID) 10434 (5'-GAGTGGCTTTGTTTCCGCTTGGATACGCCTGTCGTCCTTCACATTTGCATTCGCTTTGGTATTTCTGATC-3') and 418 (5'-GACCCTCTTAACGAATACAACGACGATCAGATCTGGGAGTCCTTGGATAACTGTCAGCTGGGAGATGAGG-3') (see Appendix A). Additional ESTs were acquired from the wheat dbEST database from NCBI (Boguski *et al.*, 1993) and assembled using the *Oryza sativa ABCC3* (*OsABCC3*; Os01g07870) as a scaffold.

Fragments of *TtABCC3* (#1-5) were generated using the OneStep RT-PCR kit (QIAGEN, Mississauga, ON, Canada). Reactions contained 250 ng RNA (combined SF-L/H pool, Pool#1), 6 μM of each primer (Table 2.2), 20 U RNaseOUT (Invitrogen, Burlington, ON, Canada) and had the cycling parameters 50°C/30m, 95°C/15m, 30×(94°C/30s, 57°C/30s, 72°C/1m), 72°C/10m. Products were purified by QIAquick PCR Purification kit (QIAGEN), and fluorescence-based cycle sequencing was performed using the BigDye Terminator v3.1 kit (Applied Biosystems, Foster City, CA, USA) with the conditions 25×(96°C/30s, 50°C/15s, 60°C/2m), and quantified on an ABI 3730 DNA Analyzer (Applied Biosystems). The raw sequences were assembled into a contig using the Contig Express module from Vector NTI Suite v10.1 (Invitrogen).

2.2.4. RCA-RACE

To obtain the remaining sequence of *TtABCC3*, RCA-RACE (Polidoros *et al.*, 2006) was used with the following modifications. Briefly, total RNA was extracted from 400 mg SF-L frozen root tissue using TRIzol reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. First-strand cDNA was synthesized from 13 μg RNA and 200 U Superscript II polymerase (Invitrogen), 40 U RNaseOUT (Invitrogen), and 0.5 μM 5'-phosphorylated primer (Table 2.2). This primer was nested within fragment TtABCC3#5 to target the remaining unknown sequence of the 5'-end of *TtABCC3* and to increase specificity to *TtABCC3*. The reaction was incubated at 42°C for 1 h, followed by RNA degradation with 2 U RNase H (Invitrogen) at 37°C for 15 min. Following purification with QIAquick PCR Purification kit (QIAGEN, Mississauga, ON, Canada), the cDNA was circularized using 150 U CircLigase (Epicentre, Madison, WI,

USA) at 60°C for 1 h, followed by heat-inactivation at 80°C for 10 min. Rolling circle amplification was performed with 1 U Φ29 DNA polymerase (New England BioLabs, Ipswich, MA, USA), 2 ng μL^{-1} bovine serum albumin, 1 mM dNTPs, and 10 μM random hexamers (phosphorothioate blocked 3'-end). Samples were incubated at 30°C overnight (~20 h) followed by heat-inactivation at 70°C for 10 min. Serial dilutions (10^{-1} to 10^{-4}) were prepared for inverse PCR (iPCR), and the original RCA reaction was stored at -20°C. iPCR was performed using 1 U Taq DNA Polymerase (QIAGEN), 0.5 μM of each primer (Table 2.2), and 2 μL diluted RCA reaction in the presence/absence of 10% dimethyl sulfoxide (DMSO) and PCR parameters of 94°C/3m, 35×(94°C/30s, 56°C/30s, 72°C/1.5m), 72°C/5m. The products were separated by agarose gel electrophoresis and the largest distinct band was excised, purified using QIAquick Gel Extraction kit (QIAGEN), and sequenced as described in Section 2.2.3.

2.2.5. *TtABCC3* Isolation and Cloning

Using 4.5 ng SF-L (Pool #1) total RNA, first-strand cDNA synthesis was carried out using 200 U Superscript III reverse transcriptase (Invitrogen, Burlington, ON, Canada), 40 U RNaseOUT (Invitrogen), and 1 μM *TtABCC3*-specific primer (Table 2.2). The reaction was incubated at 55°C for 1 h, followed by RNA degradation using 2 U RNase H (Invitrogen) at 37°C for 15 min. The full-length coding region of *TtABCC3* was amplified using the Expand Long Range kit (Roche, Laval, QC, Canada) with 0.3 μM TtABCC3&XhoI primers (Table 2.2), 10% DMSO, and 0.7 μL Enzyme Blend. Primers contained 5'-end XhoI recognition sequences for future sub-cloning. Long range PCR was performed by 95°C/2m, 15×(94°C/10s, 59°C/1m, 68°C/5m), 20×(94°C/10s,

59°C/1m, 68°C/5m20s + 20s/cycle), 68°C/5m, and subsequently purified using QIAquick PCR Purification kit (QIAGEN, Mississauga, ON, Canada). *TtABCC3* was TA-cloned using the pGEM-T vector system (Promega, Madison, WI, USA) as outlined by manufacturer. Ligation was performed at 4°C overnight (~20 h) with an insert : vector ratio of 3 : 1. The ligated products were transformed into JM109 competent cells using 10% of the mixture and cells were grown overnight on plates containing 100 mg L^{-1} ampicillin. Successful transformants were identified by blue/white colony selection and colony PCR. Seven independent colonies were selected and plasmids (pGEM-TtABCC3) were isolated using QIAprep Spin Miniprep kit (QIAGEN). All seven inserts (three *TtABCC3α*, four *TtABCC3β*,) were sequenced to determine a coding region consensus (see Section 2.2.3).

To further illustrate the requirement of DMSO for *TtABCC3* amplification, 5 ng pGEM-TtABCC3 plasmid was tested with 0 to 10% DMSO in a PCR reaction of 95°C/2m, 30×(95°C/30s, 57°C/30s, 68°C/45s), 68°C/5m. Primers targeted the first 477 bp of the 5'-end of the *TtABCC3* clone (Table 2.2).

2.2.6. Bioinformatics

Multiple sequence alignments among plant ABCCs was performed with MAFFT v6.24 (Katoh *et al.*, 2002; Katoh and Toh, 2008). Phylogenetic analysis of the MAFFT alignment was completed using the NJ algorithm (Saitou and Nei, 1987), and visualized using Phylodraw v4.0 (Choi *et al.*, 2000). Putative transmembrane regions were identified using ConPred II (Arai *et al.*, 2004). Putative domain boundaries were defined for TtABCC3 by searching against the NCBI conserved domain database (CDD;

Marchler-Bauer *et al.*, 2005). Protein localization was predicted using WoLF PSORT (http://wolfpsort.org; Horton *et al.*, 2007).

2.3. Results

2.3.1. Plant Cd Analysis

To verify segregation of the low/high NIL genotypes, shoot Cd concentration was measured in the SF-L/H and TL-L/H seedling pairs. Accumulation of Cd in shoots between each L/H isoline was significantly different ($P < 0.01$; Table 2.3). The mean Cd concentration was 43% greater for SF-H than SF-L, and 47% greater for TL-H than TL-L. Seedling pairs were pooled (3-4 pairs) to generate four independent samples. Samples with shoot Cd concentrations above the upper limit or below the lower limit of the 99% confidence interval were excluded from tissue pools for the low-Cd or high-Cd pairs, respectively.

2.3.2. qRT-PCR validation of *TtABCC3* microarray expression data

Independent techniques are required for post-analysis validation of microarray transcriptome profiling (Chuaqui *et al.*, 2002). For *TtABCC3*, qRT-PCR was used to confirm the differential expression seen in the microarray. The 200 bp amplicon used for qRT-PCR was 3'-anchored to the microarray UID10434 70-mer. The qRT-PCR results confirmed that *TtABCC3* expression was higher in the low-Cd lines than the high-Cd lines (Table 2.4), although the microarray TL Pool#2 had no difference in *TtABCC3* expression between the isolines. Comparatively, the $\log_2$ values ranged from -0.03 to 0.52

(microarray) and 0.11 to 1.23 (qRT-PCR) and the qRT-PCR mean $\log_2$ ratios were double that of the microarray ratios.

2.3.3. *TtABCC3* Isolation

Both microarray 70-mers corresponding to *TtABCC3* (UID10434 and UID418; Appendix A) were BLAST searched against the DFCI *T. aestivum* gene index release 10.0 (http://compbio.dfci.harvard.edu/tgi) to acquire their TC sequences, TC258389 and TC237754, respectively. Since both 70-mers had common microarray expression profiles and annotations, the TCs were BLAST2 (Tatusova and Madden, 1999) aligned against each other. The alignment had an overlapping region of 333 bp at 93% identity, which does not meet the 94% identity criteria required for EST clustering in the DFCI Gene Index Project. To confirm that both microarray 70-mers belong to the same gene product, a fragment spanning both TCs was amplified, and designated TtABCC3#1.

To gather additional sequence information for *TtABCC3*, *OsABCC3* was MEGABLAST searched against the wheat EST database from NCBI (~600,000 ESTs when accessed on September 17th, 2006; Boguski *et al.*, 1993), and highly similar sequences (≥90% identity; e^{-25} cutoff) were assembled (Figure 2.2). These ESTs were predominately at the 3'-end; however, homology of ESTs was low in the 3' untranslated region (UTR) of *OsABCC3*. An assembly was constructed using *OsABCC3* as a scaffold, followed by subsequent MEGABLAST searches with assembled ESTs to add additional singleton ESTs that had >90% identity to the pre-existing assembly. Gaps in the assembly and low-quality sequence overlaps amongst the EST collection required the construction of a contig from sequenced fragments to ensure an accurate *TtABCC3*

sequence (Figure 2.3). Overlapping fragments ranged in size from 635 to 1320 bp and covered 90% of the proposed *TtABCC3* coding sequence (based on *OsABCC3*), leaving approximately 500 bp unknown. Sequencing of these fragments yielded several nucleotide positions with multiple base calls (designated as a 'N' in sequence), indicating there was nucleotide variations in the PCR pool at these positions (Figure 2.4).

Strategies to obtain the remaining 5'-end sequence of *TtABCC3* include homology-based primer design (using *OsABCC3*), 5'-RACE (Frohman *et al.*, 1988), LA-PCR (Troutt *et al.*, 1992; Ansari-Lari *et al.*, 1996), and RCA-RACE (Figure 2.1; Polidoros *et al.*, 2006). Of these techniques, RCA-RACE was used to successfully isolate the remaining sequence (Figure 2.5AB). The iPCR reaction generated a distinct band of approximately 1.2 kb in samples that contained DMSO (see arrowhead in Figure 2.5B). Sequencing of the fragment identified the missing sequence (490 bp) from the 5'-end of *TtABCC3* (Figure 2.5C). This sequence had a GC-content of 73.9% and the flanking sequences matched exactly to the TtABCC3#5 fragment, including numerous N's previously identified.

To isolate the full-length coding region of *TtABCC3*, primers were designed within the UTRs by combining the EST-contig assembly and RCA-RACE sequence. These primers amplified a ~4.7 kb product (Figure 2.6A), but only in the presence of 10% DMSO. After the product was cloned, and seven independent clones sequenced, two variants of *TtABCC3* were identified (Figure 2.6BC), herein designated as *TtABCC3α* (4491 bp) and TtABCC3*β* (4503 bp). In addition, ongoing fine mapping of *TtABCC3* has shown that *TtABCC3α* and *TtABCC3β* are located on chromosomes 3A and 5B, respectively (C.J. Pozniak, University of Saskatchewan, personal communication),

indicating they are non-homeologous genes. These variants have 97% sequence homology, with 120 nucleotide differences (2.7%) and a 12 bp insert in *TtABCC3β* (at position 334 bp; see Figure 2.6B). Both *TtABCC3*s have 82% homology to both *OsABCC3* and *TaABCC2*, but only 56% homology to *AtABCC3*. The overall GC-content is 54% and 55% for *TtABCC3α* and *TtABCC3β*, respectively; however, the 5'-end is GC-rich compared to the entire coding region (Figure 2.7A). In addition, the GC profile of *TtABCC3* resembles that of the *OsABCC3* as opposed to *AtABCC3*. The requirement of DMSO for *TtABCC3* isolation is probably due to the high GC content at the 5'-end, as this region could only be amplified with ≥5% DMSO concentrations (Figure 2.7B).

2.3.4. TtABCC3 Protein Structure

TtABCC3α and TtABCC3β encode proteins of 1496 and 1500 amino acids, respectively (Figure 2.8AB), and share 97% amino acid similarity with 36 amino acids that differ between them (excluding the 4 residues from the TtABCC3β insert). The predicted molecular weight and isoelectric point are 163 kDa and 5.94 for TtABCC3α, and 164 kDa and 6.02 for TtABCC3β. Both have protein similarity of 82% to OsABCC3, 78% to TaABCC2, and 61%, to AtABCC3.

TtABCC3 is predicted to contain 13 transmembrane regions (Figure 2.8C). The conserved TMD-NBF-TMD-NBF domain arrangement of eukaryotic ABC transporters is maintained, and TtABCC3 also has the characteristic NTE domain of the ABCC subclass (Figure 2.8; Tusnády *et al.*, 1997). Within each NBF, the domain possessed (in order) the Walker A, ABC signature, and Walker B motifs (Figure 2.9) – which were identical for each variant. WoLF PSORT (Horton *et al.*, 2007) predicted both TtABCC3α and

TtABCC3β to localize to the plasma membrane. Phylogenetic analysis of several identified plant ABCCs (Figure 2.10) showed TtABCC3 belongs to a distinct cluster of ABCCs from monocots (OsABCC3-5, TaABCC2, TtABCC3, and ZmABCC2), and is separate from a clade of dicots (AtABCC3, 7 and 8).

2.4. Discussion

The full-length coding region of *TtABCC3* was isolated from durum wheat by combining sequencing information from an EST-based contig and RCA-RACE. Sequence analysis identified two distinct versions of *TtABCC3* – *TtABCC3α* and *TtABCC3β* – which were independently found to be non-homeologous genes (C.J. Pozniak, University of Saskatchewan, personal communication). These are the first non-homeologous ABCCs isolated from a polyploid. Sequencing of the contig fragments also supported the presence of two distinct transcripts, because multiple base calls were comprised of two individual nucleotide base calls from *TtABCC3α* and *TtABCC3β* (see Figure 2.4 as an example). To negate the possibility that the polymorphic bases were due to cross-contamination of RNA from different isolines during sample pooling (i.e. combining a low-Cd with high-Cd isoline), individual SF-L/H plants were sequenced. Even with sequences from individual plant, the polymorphic base calls were retained. Given that durum wheat is tetraploid ($2x = 4n = 28$; AABB genome), isolation of two variants of *TtABCC3* (one per genome) might be expected. Comparison of consensus sequences to the original EST collection revealed α- and β-specific EST clusters (Figure 2.4), supporting the variants cloned from durum wheat. Furthermore, a separate grouping of ESTs matched neither *TtABCC3* variant, and most likely originates from ABCC3 on

genome D, since the majority of EST datasets were derived from *T. aestivum* ($2x = 6n = 46$; AABBDD genome).

I hypothesized that TtABCC3 would sequester Cd into the vacuole of root cells of durum wheat, thereby limiting root-to-shoot translocation. Of the two TtABCC3 variants, TtABCC3β may be most relevant to this hypothesis, since *TtABCC3β* maps to chromosome 5B, the region that is associated with inheritance of the low-Cd accumulation trait (Knox *et al.*, 2003). Deciphering how much each variant contributes to the differential expression of *TtABCC3* could address whether *TtABCC3β* has higher expression in the low-Cd isoline. Unfortunately, the high sequence homology shared by both *TtABCC3*s would prevent the microarray probes from hybridizing to unique transcripts, even though the probes are not identical to either gene (see Appendix A and Figure 2.6BC). A more sensitive and stringent approach like qRT-PCR could be used to quantify expression levels of the α and β variants, by exploiting the 12 bp insert found in *TtABCC3β*. However, this insert region is GC-rich and could be difficult to amplify under qRT-PCR chemistry. GC-rich templates can form intramolecular stem loops when single-stranded, resulting in shortened PCR product as the DNA polymerase jumps the hairpin structure (Viswanathan *et al.*, 1999). An alternative technique makes use of molecular beacon oligonucleotides that can select for one (or more) of the polymorphic bases between the variants that is located downstream from the GC-rich 5'-end (Mhlanga and Malmberg, 2001).

Results from both qRT-PCR and microarray illustrated that *TtABCC3* expression was higher in the low-Cd isoline. Discrepancies between the two data sets may be attributable to increased stringency and sensitivity found in qRT-PCR reactions (Kubista

et al., 2006). During microarray hybridization, individual spots can interact with both target and non-specific cDNAs, usually (but not always) due to a common motif within the 70-mer sequence (Choi *et al.*, 2000). Non-specific hybridization can ultimately lead to the 'true' transcript signal being masked or make the spot appear differentially expressed. Such mis-hybridization is reduced in qRT-PCR as primers and reaction parameters (melting temperature, magnesium concentration, etc.) can be optimized to yield one amplicon corresponding to one gene.

Amplification of the full-length *TtABCC3* sequence required the addition of the co-solvent DMSO (Figure 2.6A), and is probably due to the GC-rich sequence at the 5'-end of the gene (Figure 2.7A). Moreover, the requirement of DMSO for amplification may also explain the lack of ESTs in this region (Figure 2.2). DMSO is thought to destabilize intramolecular secondary structure of single-stranded template (Pomp and Medrano, 1991), thus increasing the processivity of DNA polymerase. Shen *et al.* (1992) reported DMSO enhanced the amplification of complex secondary structures using Klenow and Taq polymerase-mediated reactions. DMSO has also been shown to be beneficial to cDNA synthesis during RT-PCR, possibly by eliminating non-specific amplification (Sidhu *et al.*, 1996). *TtABCC3* amplification used a two-step approach where the RT and PCR reactions were separated, and only the PCR reaction contained DMSO. Thus, DMSO was not required for cDNA synthesis. This suggests that DMSO improves the amplification of intramolecular secondary structure found in the GC-rich sequence. DMSO-dependent amplification of the plasmid pGEM-TtABCC3 further indicates a PCR-specific effect on the DNA polymerase (Figure 2.7B), although an affect of DMSO on reverse transcription cannot be ruled out.

The GC-rich 5'-end is not unique to *TtABCC3* or even wheat. Theodoulou *et al.* (2003) suggested that high-GC sequence prevented the isolation of the full-length coding region of *TaABCC1*. Surveys of gene composition between *Gramineae* species (monocots) and eudicots revealed that monocots had a gradient of GC content that was negatively correlated with transcription length (Wong *et al.*, 2002). Generally, the 5'-end was up to 25% more GC-rich than the 3'-end, a similar trend to that observed in *TtABCC3* and *OsABCC3*, but not *AtABCC3* (Figure 2.7A). This would also suggest that monocot *ABCC*s have unique NTE amino acid compositions not seen in dicots, as these genes would use different codons during translation. Since NTEs are known to play a role in protein trafficking (Mason and Michaelis, 2002; Westlake *et al.*, 2005), ABCCs derived from monocots may have unique membrane localization signals.

Both *TtABCC3* genes encoded proteins that contained typical features of ABCC transporters, including the number of transmembrane spans, domain organization, conserved motifs and the presence of the NTE (Figure 2.8 and Figure 2.9). Differences in amino acid residues between TtABCC3α and TtABCC3β may impact the protein function; however, none of these differences alter the ABCC conserved motifs. Plant ABCCs are known to transport a wide variety of substrates and with different affinities (Klein *et al.*, 2006). Conformational changes in protein structure due to amino acid substitutions could affect the interaction of residues with substrates, thereby altering substrate affinities and binding. For instance, single site-directed mutations of conserved glycine residues in the Walker A motifs (indicated in bold: **G**X_2GXGK[S,T]) of YCF1 abolished glutathione-conjugate transport and subsequently Cd tolerance (Falcón-Pérez *et al.*, 1999).

TtABCC3α and TtABCC3β are both predicted to localize to the plasma membrane rather than to the tonoplast, suggesting TtABCC3 may export Cd from the cytoplasm instead of sequestering Cd into the vacuole. However, the WoLF PSORT algorithm used to predict subcellular localization of TtABCC3α/β has a low representation for organelles such as the vacuole (Horton *et al.*, 2007). For instance, the known vacuolar transporters AtABCC1 (Geisler *et al.*, 2004) and AtABCC2 (Liu *et al.*, 2001) both annotate as localizing to the plasma membrane. These results highlight the inaccuracy of WoLF PSORT (and other bioinformatic prediction tools) and demonstrate that TtABCC3 could still be localized to the tonoplast.

To deduce if TtABCC3 is involved in sequestration of Cd into the vacuole, each variant will be heterologously expressed in the Cd-hypersensitive *ycf1* yeast mutant (Szczypka *et al.*, 1994; Li *et al.*, 1996) to see if Cd tolerance is restored. The transport rates may differ between TtABCC3α and TtABCC3β, and if so, could provide a unique scenario to identify critical residues (out of the 40 differences) that might control the transport of Cd.

Table 2.1. Total concentration, free activity and free concentration of ions, ligands, and buffers of the chelator-buffered nutrient solution. Chemical speciation was calculated using GEOCHEM-PC.

Chemical Species	Total Concentration (μM)	Free Activity (μM)	-log Free Activity	Free Concentration (μM)	-log Free Concentration
Ca^{2+}	1000	687	3.16	957	3.02
Mg^{2+}	300	211	3.68	294	3.53
K^{+}	2120	1950	2.71	2120	2.68
Na^{+}	0.4	0.368	6.43	0.400	6.40
Fe^{3+}	100	2.28×10^{-11}	16.64	4.81×10^{-11}	16.32
Mn^{2+}	1	0.018	7.75	0.025	7.61
Cu^{2+}	2	3.62×10^{-8}	13.44	5.05×10^{-8}	13.30
Cd^{2+}	0.5	1.44×10^{-5}	10.84	2.00×10^{-5}	10.70
Zn^{2+}	10	1.14×10^{-4}	9.94	1.60×10^{-4}	9.80
Ni^{2+}	0.1	3.61×10^{-9}	14.44	5.03×10^{-9}	14.30
SO_4^{2+}	113	71.4	4.15	99.6	4.00
Cl^{-}	51.2	47.1	4.33	51.2	4.29
NH_3	300	0.276	6.56	0.276	6.56
PO_4^{3-}	100	2.29×10^{-6}	11.64	4.83×10^{-6}	11.32
$B(OH)_4^{-}$	10	6.27×10^{-3}	8.20	6.82×10^{-3}	8.17
MoO_4^{2-}	0.2	0.141	6.85	0.197	6.71
NO_3^{-}	3450	318	2.50	3450	2.46
MES	2000	552	3.26	600	3.22
HEDTA	139	1.01×10^{-5}	11.00	2.13×10^{-5}	10.67

Table 2.2. Sequences and properties for primers used for qRT-PCR and isolation of *TtABCC3*.

Primer[1]	T_m (°C)	%GC	Complementary Score[2] Any	Complementary Score[2] 3'	Sequence (5'→3')
***TtABCC3* qRT-PCR**					
TtABCC3q200-S	59.8	55.0	4	1	AGAGCTGCAAAGGCTGGTAG
TtABCC3q200-AS	60.2	45.0	3	2	TATCCAAGCGGAAACAAAGC
Actin-S	59.9	52.4	3	4	GGTGAAGGGGACTTACAAAGG
Actin-AS	60.0	50.0	2	0	TTCATACAGCAGGCAAGCAC
***TtABCC3* contig**					
TtABCC3#1-S	59.6	50.0	6	2	CTGTTGCAAGTTCGCTATGC
TtABCC3#1-AS	57.2	52.4	3	3	CCCAGCTGACAGTTATCCAAG
TtABCC3#2-S	60.0	55.0	5	0	ATGACAGGGTCCTTGAGTGG
TtABCC3#2-AS	60.2	45.0	3	2	TATCCAAGCGGAAACAAAGC
TtABCC3#3-S	60.4	50.0	4	0	CCAACGATGTTTGAGGGAAC
TtABCC3#3-AS	59.9	55.0	6	2	TACCGTGCGCGTATCAGTAG
TtABCC3#4-S	60.3	52.4	6	2	TCTCCGTCGTGTACCAGAAAG
TtABCC3#4-AS	61.9	50.0	6	2	CTTGAAAAGGTGGGATCCTG
TtABCC3#5-S	60.4	63.2	2	0	CGCTCTTCCTGCTCCTCTC
TtABCC3#5-AS	60.6	55.0	3	2	GTCGCCTTCATCCTGACATC
RCA-RACE					
RCA-RACE-AS[3]	60.6	55.0	3	2	*(P)*-GTCGCCTTCATCCTGACATC
iPCR-S	60.3	52.4	6	2	TCTCCGTCGTGTACCAGAAAG
iPCR-AS	60.8	55.0	4	2	CTCCAGGTTCGTCTTGAACG
***TtABCC3* full-length**					
TtABCC3-AS	60.9	50.0	4	0	CGAGAGCACTTCACATCACATC
TtABCC3&XhoI-S[4]	69.2	72.2	5	2	**CTCGAG**ACCCAGCCTGCGCCCATC
TtABCC3&XhoI-AS[4]	64.3	38.5	4	2	**CTCGAG**ACCAGTAATTTTTCGGCATTATCGAC
DMSO 5'-end analysis					
DMSO-S	69.2	72.2	5	0	ACCCAGCCTGCGCCCATC
DMSO-AS	60.6	60.0	8	2	TACTGGAGCTGCAGGTAGGC

[1]Primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; Rozen and Skaletsky, 2000). [2]Local alignment score for self-complementation based on any allowable annealing (Any) or 3'-anchored annealing (3'). Scoring system: +1 Complementary; -1 Mismatch; -2 Gap. Lower is better. [3]Primer is 5'-phosphorylated, which is required for CircLigase-mediated cDNA circularization. [4]Primer designed with XhoI cut-site on 5'-end (indicated in **bold**).

Table 2.3. Shoot cadmium concentrations (mg kg^{-1}) of 21-d-old SF and TL near-isogenic durum wheat seedling pairs prior to pooling for RNA extraction.

Bucket[1]	SF-L	SF-H	TL-L	TL-H
1	**0.94**	1.08	0.68	1.02
2	0.71	0.96	0.63	1.04
3	0.70	1.14	0.58	0.89
4	**1.04**	**0.87**	0.42	0.88
5	0.52	0.93	0.60	0.99
6	0.55	**0.81**	0.50	0.83
7	0.73	**0.83**	0.71	**0.57**
8	0.68	1.15	0.55	0.82
9	0.62	1.03	**0.88**	1.46
10	0.82	1.63	**0.89**	0.91
11	0.62	1.18	0.61	1.01
12	**0.90**	1.09	**0.88**	1.22
Mean ± SD	0.74 ± 0.16	1.06 ± 0.22	0.66 ± 0.15	0.97 ± 0.22
Student's *t*-test	<0.001		<0.001	
99% Confidence Interval[2]	0.62 - 0.86	0.89 - 1.22	0.54 - 0.77	0.81 - 1.13

[1]Each sample represents two seedlings per bucket; combined at harvest.
[2]Individual samples were excluded from composite pools (as indicated in **bold**) if the low-Cd or high-Cd sample was above the upper limit or below the lower limit of the 99% confidence interval, respectively.

Table 2.4. Comparison of microarray and qRT-PCR results for *TtABCC3* expression from TL and SF near-isogenic lines.

	TL Pools[1]				SF Pools				
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**Mean (SE)**
Microarray	0.41[†]	-0.03	0.54	0.26	0.52	0.29	0.27	0.23	0.31 (0.07)
qRT-PCR	0.71	0.56	0.73	1.23	0.63	0.52	0.77	0.11	0.66 (0.11)

[1] Pools represent 2-3 pairs (4-6 seedlings) combined after Cd analysis of shoot tissue.
[†] Numbers are $\log_2$ ratios of low-Cd/high-Cd.

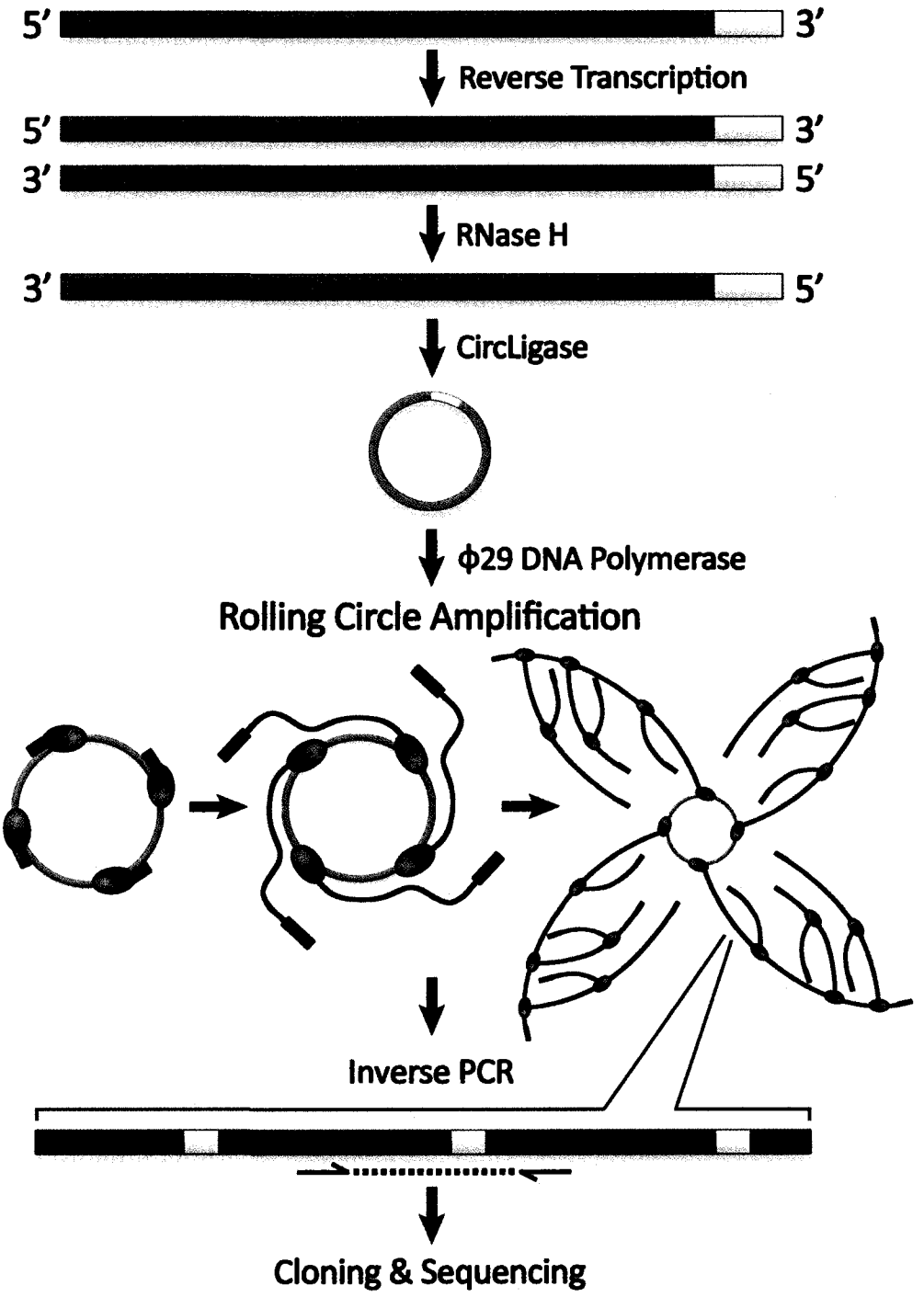


Figure 2.1. Overview of RCA-RACE.
Total RNA or mRNA (**blue**) is reverse transcribed into cDNA (green) using either an oligo(dT) or gene-specific primer (**white** section). Following degradation of RNA with RNase H, the cDNA is circularized by CircLigase and amplified using ϕ29 DNA polymerase (**violet**) and random hexamers (**red**) to yield multi-copy concatamers (**black**). Target transcripts are re-amplified from the RCA pool by inverse PCR, cloned and sequenced. Adapted from Polidoros *et al.* (2006).

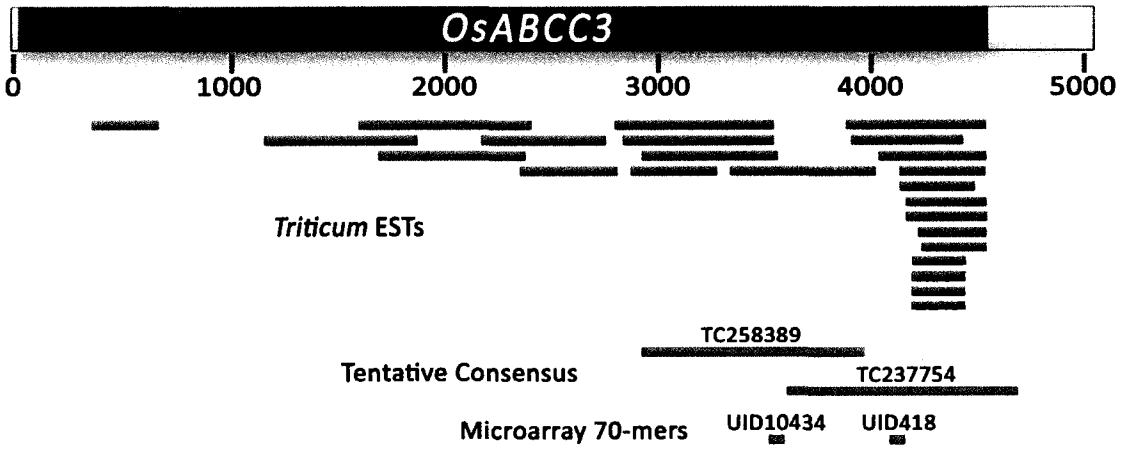


Figure 2.2. ESTs identified by *OsABCC3* MEGABLAST.
Using the *OsABCC3* mRNA (Os01g07870; **black** section is coding region) as a probe, the NCBI *Triticum* EST database (http://www.ncbi.nlm.nih.gov/dbEST; Boguski *et al.*, 1993) was searched for highly similar ESTs (**red**) using MEGABLAST (≥90% identity; e^{-25} cutoff). Microarray 70-mers (**green**) and their respective tentative consensus (TC) sequences (**violet**) are also shown. Access date: September 17th, 2006.

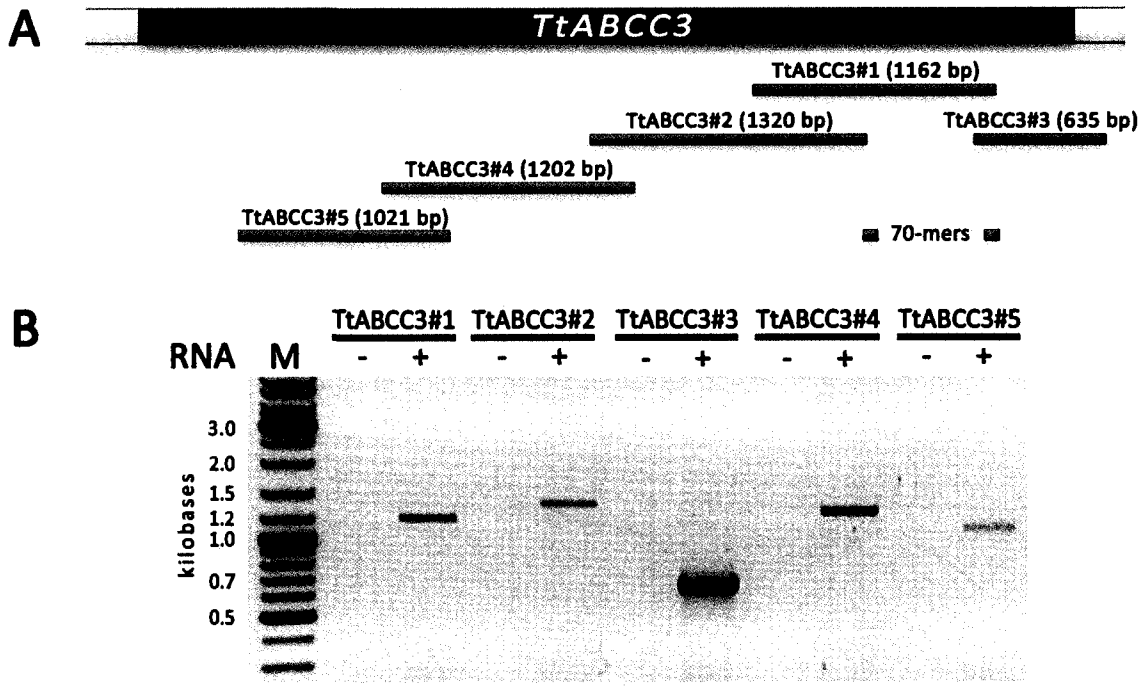


Figure 2.3. *TtABCC3* sequencing contig.
(A) Diagram. Graphical representation of the five fragments (TtABCC3#1-5; **blue**) amplified to construct the *TtABCC3* contig. Microarray 70-mers (**green**) are shown for relative comparison to Figure 2.2. The *TtABCC3* gene model is shown with coding region (**black**) and untranslated regions (**white**). **(B) RT-PCR amplification of *TtABCC3* fragments.** Fragments were amplified with/without SF-L RNA through RT-PCR, and gel-analyzed. Purified PCR products were sequenced directly. Similar results were obtained for SF-H RNA (data not shown). M, Generuler DNA Ladder Mix.

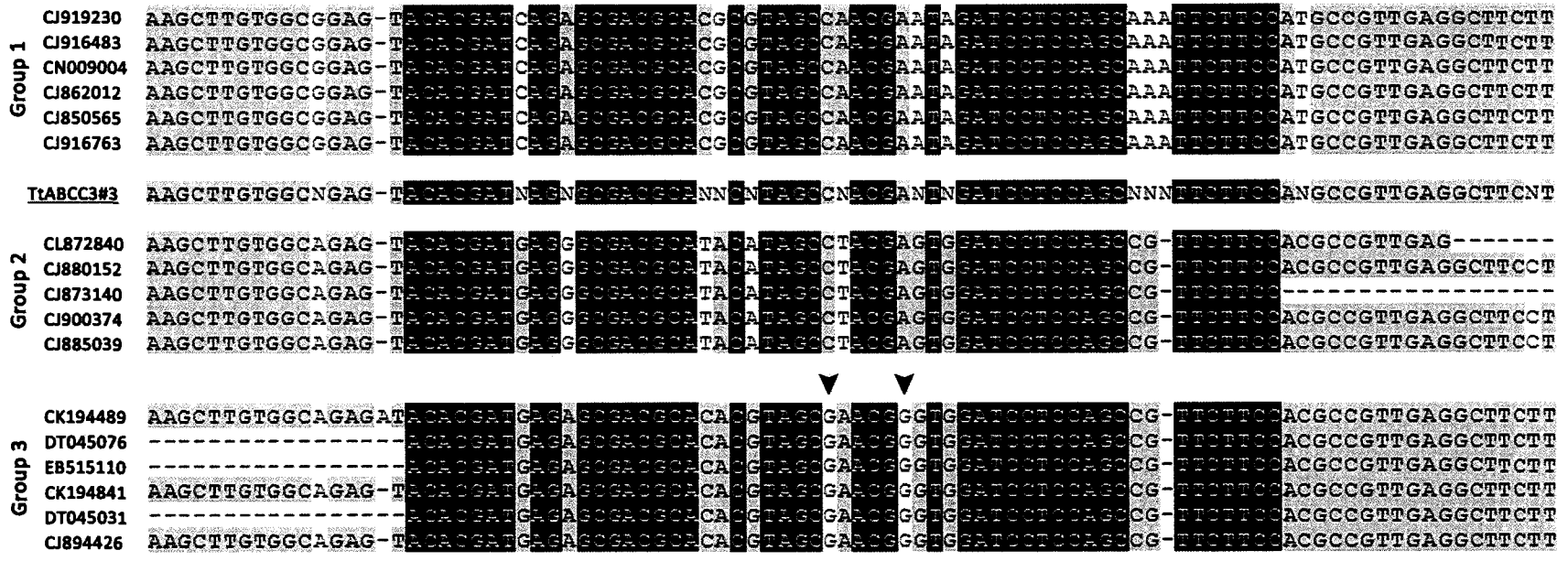
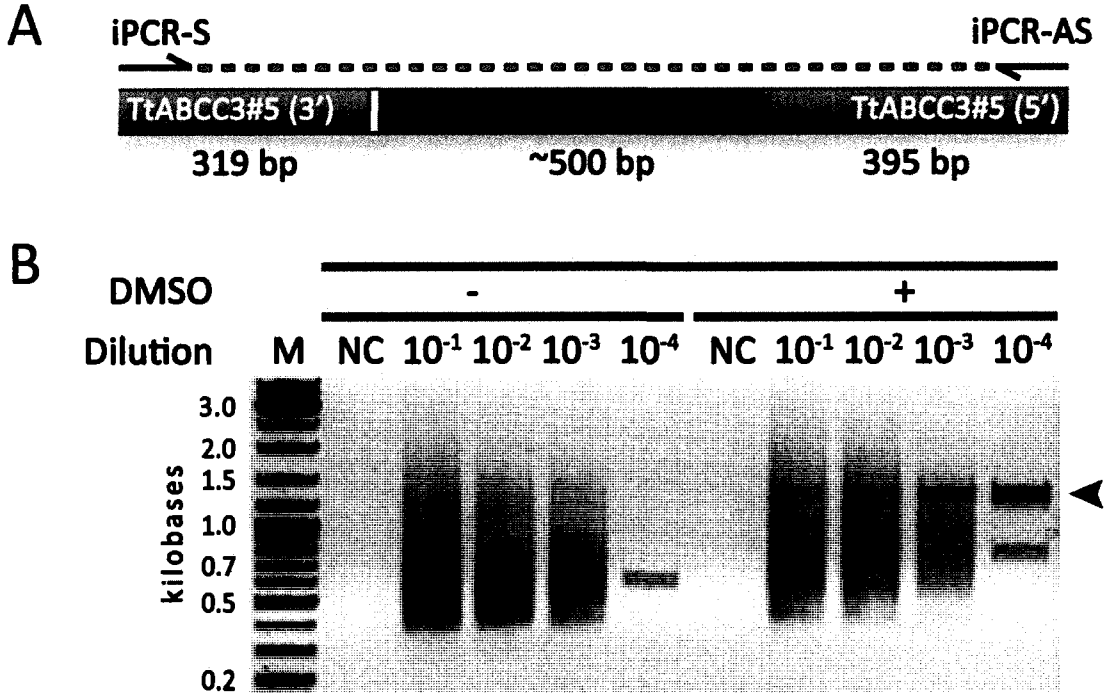


Figure 2.4. Grouping of ESTs based on TtABCC3#3 sequence.
Nucleotide alignment of ESTs with a segment of the TtABCC3#3 sequence using the AlignX module from Vector NTI Suite v10.1 (Invitrogen). Based on sequence variation at nucleotide positions, three distinct groups of ESTs were designated. Identical and weakly similar regions are highlighted in **black** and gray, respectively. Arrowheads indicate nucleotide positions that were not polymorphic in *TtABCC3*, but were in Group 3 ESTs. NCBI accession numbers for ESTs are shown to the left of the sequence. N, multiple base call.



C

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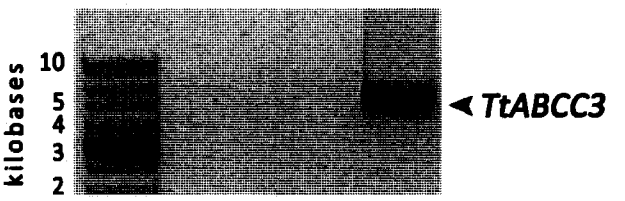
...GTTCATCTTGTACTCCACCCTGGGGGTAGCGTCGCTTGCNGCGCTCGGTGCCACCATTGTGGTCATGCTTGCAAATGTGCCTCCCAT
GAAAATGCAGGAGAAGTTCCAGCAGAAGCTCATGGACTGCAAGGATGTCAGGATGAAGGCGAC

1   ACTCACCCAGCCTGCGCCCNTCCATGCCCACCGCGGCGTCGNCGTCGTCGTCNCCTTTCGCGGCAGCGATGGCGGAGCCGGCCGCCCTCC
91  CCGTCTTCCTGCGGCCGCTTCTCCTCCACGGCCTCGGCGCCGGGGCCCACCTCCTGCTCGCACTCGCCGTCGCGGCCCGCCTCCTCTTCG
181 CCNCCGCCCNCCGCGGCAAGGAATCAGACNGCTGCGGCGAGAGGCGGCCGCGCCGGGTTCCGGTGGGGCCTGTTCGCGGTGCGCGCCACG
271 TGGGCTCTGGCGGCGTNCGAGGTCTTCCTCGNCGNGTACTCGCTCGTCTCGNGNNNCCTCNACAACAGCGGGNCCGGGTGGGGGGCGCCC
361 GACGCGGTGGCCGACCAGGCGGACNCCGCGGCGCGCGCGGTGGCGTGGCTGCTGCTCGCGGCCTACCTGCAGCTCCAGTACCGNAGCCNC
451 GGGGAGGAGCGGTTCGCCGCGCCGCTCAAGCTCTGGTGGG

    CGCTCTTNCTNCTCCTCTCCGTNCTCGCNCTCGCCGTCCACGCCGCNACGAGCCTCTNCTACGGGNTCCCCGTGCCCGCGCTTCCGTGGG
    CGCGCGACGCCGTTGAGGTCCTCGCAGNCGTGGCGCTAC...
```

Figure 2.5. Use of inverse PCR to obtain 5'-end of *TtABCC3*.
(A) Diagram of iPCR reaction. Dotted line (**red**) illustrates product region that spans from the 3'-end of TtABCC3#5 (**blue**), across the cDNA ligation junction (**white**) and through the proposed missing sequence (**black**) to the 5'-end of TtABCC3#5. **(B) Inverse PCR.** Amplification of the 5'-end of *TtABCC3* using nested primers and serial dilutions of the RCA pool. Desired band product size (~1.2 kb) was only obtained with the addition of dimethyl sulfoxide (DMSO). Arrow signifies gel band excised for sequencing. M, Generuler DNA Ladder Mix; NC, negative control. **(C) 5'-end TtABCC3 Sequence.** Sequence derived from excised gel band obtained using RCA-RACE in the presence and absence of DMSO. Black letters highlight newly acquired sequence (with coding region in **bold**), while gray letters are TtABCC3#5 fragment sequence. The RCA-RACE-AS primer used for cDNA synthesis and the TtABCC3#5-S primer are underlined in the top and bottom sections, respectively. N, multiple base calls.

A



B

```
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4231  TCTGAAACGGACCAAGATACTGGTTCTGGACGAAGCCACTGCCTCAGTGGATACCGCGACAGACAACATGATCCAGAAAACGCTGCGTGA
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4591  ATTACTGGT
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C

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721  CGATGCTTCCCTCTTCACGAGCGCCGGCTTCCTCAGCGTCCTCACCTTCTCCTGGATGGGACCCCTGCTCGCCGTCGGCAACAAGAAGGC
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1621 GAAGTACCTTTACACATCGACCGCGGCCACCTTCGTGTTCTGGGGTGCCCCGACCTTCGTCGCTGTGGTAACCTTCGGAGCTTGCATGCT
1711 CTTAGGGATACCATTGGAGTCAGGGAAGGTGCTGTCTGCATTGGCCACGTTCCGGGTGCTTCAAGAACCAATATACAACCTTCCTGACAC
1801 AATCTCGATGATGATTCAGACCAAGGTGTCTCTTGACAGGATAGCATCTTTCCTATGCCTTGAGGAGTTGCCGACGGATGCCGTGGAGAG
1891 GCTACCAAGTGGTAGCTCCAATGTTGCAATTGAGGTCAGCAATGGGTGCTTCTCCTGGGACGGCTCACCTGAGCTGCCAACGCTGAAGGA
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4591 TAATGCCGAAAAATTACTGGT
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Figure 2.6. *TtABCC3* isolation and sequence.

(A) TtABCC3 Isolation. The complete coding sequence of TtABCC3 was amplified by long-range PCR using primers located in the untranslated region, albeit only in the presence of 10% DMSO. Expected product size is 4.7 kb. M, Generuler DNA Ladder Mix; NC, negative control. (B and C) TtABCC3α and TtABCC3β sequence. Consensus nucleotide sequence of cloned TtABCC3 inserts, with the coding region highlight in bold. Polymorphisms between variants (**blue**), including 12 bp insert found in TtABCC3β (blue) are shown. Hybridization sites for 70-mers (UID 10434 and 418) are also shown (underline).

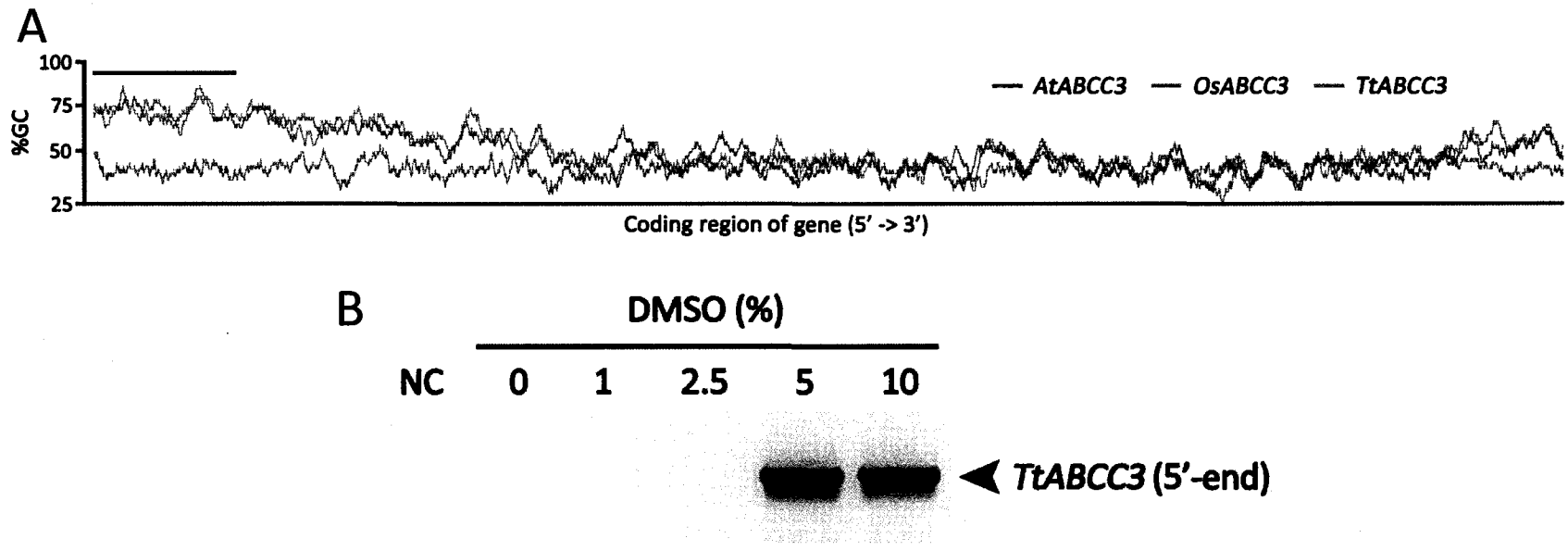

Figure 2.7. Analysis of the 5'-end of *TtABCC3*.
(A) GC profiles. Using a 51 bp window, the GC content was determined over the coding sequences of *AtABCC3* (—) *OsABCC3* (—) and *TtABCC3* (—). Both *TtABCC3α* and *TtABCC3β* gave nearly identical profiles; only *TtABCC3α* is shown. **(B) DMSO requirement for *TtABCC3* amplification.** At DMSO levels from 0 to 10%, only concentrations ≥5% amplified the 5'-end of *TtABCC3*. DMSO analysis completed by James Maclagan (Department of Biological Sciences, University of Alberta). Region amplified for DMSO studies is marked with a black line in **(A)**. NC, negative control.

A

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1    MPTAASSSSSPFAAAMAEPAALPVFLRPLLLHGLGAGAHLLLALAVAARLLFAAAPRGKESAAAARGGRAGFRWGLFAVRATWALAASEV
91   FLGAYSLVSWYLDNSGAGWGAPDAVADQADAAARAVAWLLLAAYLQLQYRSHGEERFAAPLKLWWALFLLLSVLALAVHAATSLCYGVPV
181  PALPWARDAVEVLAAVALLVAGFSAKTTGGSASEEPLLNGASESRGDDTVDASLFTSAGFLSVLTFSWMGPLLAVGYKKALGLDDVPDLD
271  HADSVAGLLPSFKTNLEAQAGDGSGPKFTAFKLTKALVRTVWWHIAVTALYALIYNLATYVGPYLIDSLVQYLNGDERYASKGKLLVVTF
361  IVAKVFECLSQRHWFFRLQQAGIRARSALVSVVYQKGLSLSSTSRQSRTSGEMINIISVDADRVGLFSWYMHDLWLVPLQVGMALFILYS
451  TLGVASLAALGATIVVMLANVPPMKMQEKFQQKLMDCKDVRMKATSEILRNMRILKLQGWEMKFLSKIIDLRTTETSWLKKYLYTSTAAT
541  FVFWGAPTFVAVVTFGACMLLGIPLESGKVLSALATFRVLQEPIYNLPDTISMMIQTKVSLDRIASFLCLEELPTDAVERLPSGSSNVAI
631  EVSNGCFSWDGSPELPTLKDLNFQAQQGMRVAVCGTVGSGKSSLLSCILGEVPKLSGEVKTCGTMAYVSQTAWIQSGKIQDNILFGKEMD
721  SEKYDRVLEWCSLKKDLEILPFGDKTVIGERGINLSGGQKQRIQIARALYQDADIYLFDDPFSAVDAHTGSHLFKECLLGALASKTVVYV
811  THQIEFLPSADLILVMKGGRIAQAGKYNDILGSGEELMELVGAHQDALTALDVIDVANGGSETISLSLSRSLSSSEEKDKQNGKDDGDKV
901  QSGQLVQEEEREKGRVGFWVYWKYLTLAYGGALVPFVLIAQLLFQVLQIASNYWMAWASPVSKDAKPPVSTSTLIYVFVALAVASSLCIL
991  IRALFLVTAAYKTATLLFNKMHMAIFRAPMSFFDSTPSGRILNRASTDQSEVDTNIAYQMGSVAFSIIQLVGIIAVMSQVAWQVFLVFVP
1081 VIIICFYYQRYYIETARELQRLVGVCKAPIIQHFAESITGSTTIRSFGKENQFVSTNSHLMDAYSRPKFYNAAAMEWLCFRLDTLSSFTF
1171 AFALVFLISLPTGIIDPGIAGLAVTYGLNLNMLQAWVVWSMCNLENKIISVERILQYISIPEEPPLTMSEDKLPHNWPSEGEIQLCDVHV
1261 RYAPQLPFVLKGLNVTFPGGMKTGIVGRTGSGKSTLIQALFRIVEPTVGQILVDGVDICTIGLHDLRSRLSIIPQDPTMFEGTVRSNLDP
1351 LNEYNDDQIWEALDNCQLGDEVRKKELKLDSPVIENGENWSVGQRQLVCLGRVILKRTKILVLDEATASVDTATDNMIQKTLRENFSEAT
1441 VITIAHRITSVLDSDMVLLLDNGVAVEHDTPAKLLENKSSLFSKLVAEYTMRATHT
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B

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1    MPTVASASSSPFAAAIAEPAALPVFLRPALLHGLGAGAHLLLALAVAARLLFAPAHRGKESAAAVRGGGIRFRWGQFAVRATWALAASEV
91   FLGVYSLVSWYLDNSGTGGGAGWGAPDAVADQADTAARAVAWLLLAAYLQLQYRGRGEERFAAPLKLWWALFLLLSVLALAVHAATSLSY
181  GLPVPALPWARDAVEVLAGVALLVAGFSANTTGGSASEEPLLNGASESRGDDTVDASLFTSAGFLSVLTFSWMGPLLAVGNKKALGLDDV
271  PDLDHADSVAGLLPSFKTNLEAQAGDGSGPKFTAFKLTKALVRTVWWHIAVTALYALIYNLATYVGPYLIDSLVQYLNGDERYASKGKLL
361  VVTFIVAKVFECLSQRHWFFRLQQAGIRARSALVSVVYQKGLSLSSISRQSRTSGEMINIISVDADRVGLFSWYMHDLWLVPLQVGMALF
451  ILYSTLGVASLAALGATIVVMLANVPPMKMQEKFQQKLMDCKDVRMKATSEILRNMRILKLQGWEMKFLSKIIDLRTTETSWLKKYLYTS
541  TAATFVFWGAPTFVAVVTFGACMLLGIPLESGKVLSALATFRVLQEPIYNLPDTISMMIQTKVSLDRIASFLCLEELPTDAVERLPSGSS
631  NVAIEVSNGCFSWDGSPELPTLKDLNFEAQQGMRVAVCGTVGSGKSSLLSCILGEVPKLSGEVKTCGTMAYVSQTAWIQSGKIQDNILFG
721  KEMDSEKYDRVLEWCSLKKDLEILPFGDKTVIGERGINLSGGQKQRIQIARALYQDADIYLFDDPFSAVDAHTGSHLFKECLLGALASKT
811  VVYVTHHIEFLPSADLILVMKGGRIAQAGKYNDILGSGEELMELVGAHQDALTALDVIDVANGGSETISSSLSRSLSSAEEKDKQSGKDN
901  GDKVQSGQLVQEEEREKGRVGFWVYWKYLTLAYGGALVPFVLIAQLLFQVLQIASNYWMAWASPVSKDAEPPVSTSTLIYVFVALAVASS
991  LCILIRALFLVTAAYKTATLLFNKMHMAIFRAPMSFFDSTPSGRILNRASTDQSEVDTNIAYQMGSVAFSIIQLVGIIAVMSQVAWQVFL
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1351 NLDPLNEYNDDQIWEALDNCQLGDEVRKKELKLDSPVIENGENWSVGQRQLVCLGRVILKRTKILVLDEATASVDTATDNMIQKTLRENF
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C

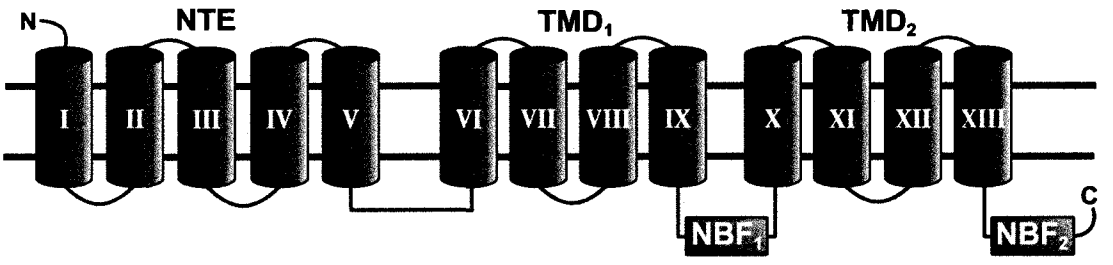

Figure 2.8. TtABCC3 protein and topology.

TtABCC3α (A) and TtABCC3β (B) protein sequence. Domains of the translated protein sequence are coloured for NTE (**red**), TMDs (**blue**) and NBFs (**green**). Within each NBF, the following ABC motifs are underlined (in order): Walker A, ABC signature motif, and Walker B. Domains were defined by the NCBI Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Marchler-Bauer *et al.*, 2005), except for the NTE, where the boundaries are from the first amino acid to the end of fifth predicted transmembrane region. Residues that differ between proteins are indicated in **bold**, and the TtABCC3β insert is highlighted (red) **(C) Membrane Topology.** Simplified diagram of transmembrane regions and domains (NTE, TMDs, and NBFs) of TtABCC3. Transmembrane spans were predicted by ConPred II (Arai *et al.*, 2004). Both TtABCC3 variants give rise to the same general topology.

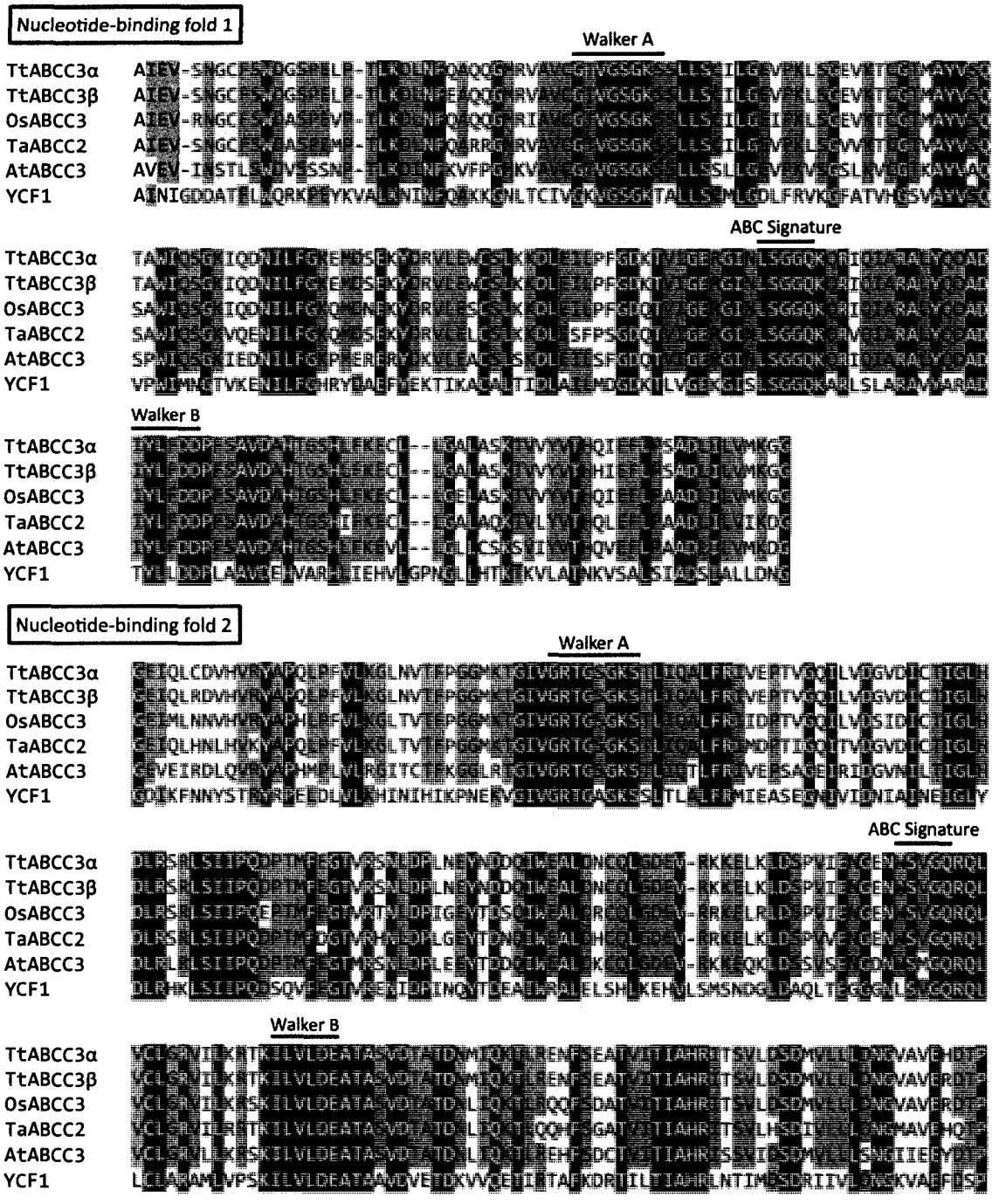

Figure 2.9. Protein alignment of the ABCC nucleotide-binding folds.
Protein alignment using MAFFT (Katoh *et al.*, 2002; Katoh and Toh, 2008) of the NBF domains for TtABCC3α, TtABCC3β, OsABCC3 (Os01g07870), TaABCC2 (AAQ10074), AtABCC3 (At3g13080), and YCF1 (NP_010419). Identical and highly similar residues are highlighted in **black** and gray, respectively. ABC motifs are identified: Walker A ($GX_2GXGK[S,T]$), ABC signature (LSSGQ), and Walker B (Φ_4DE; where Φ is a hydrophobic residue).

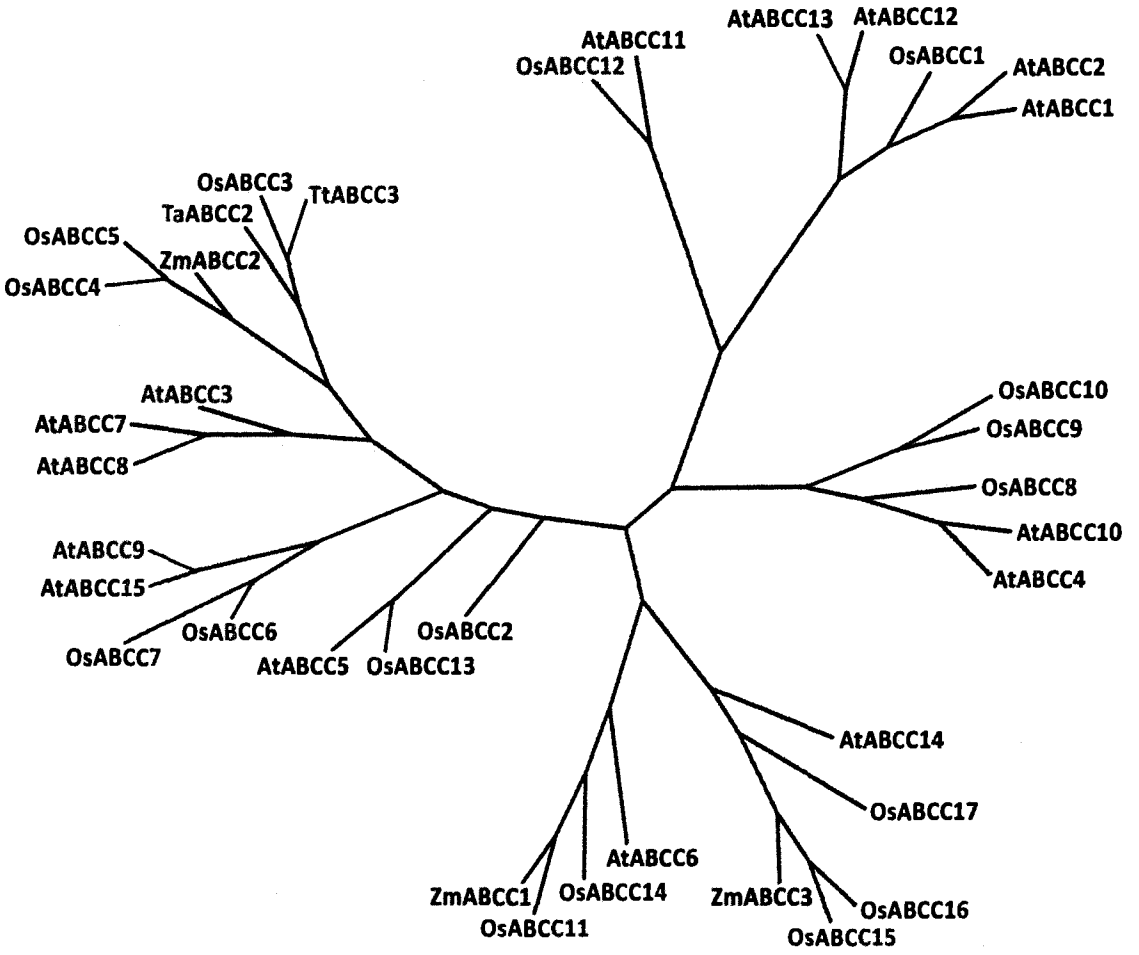

Figure 2.10. Phylogenetic analysis of plant ABCC proteins.
Unrooted tree of the predicted relationship between known Arabidopsis (**red**), rice (**blue**), maize (**violet**), and wheat (**green**) ABCCs. Alignment was constructed using MAFFT (Katoh *et al.*, 2002; Katoh and Toh, 2008), and the phylogeny tree was constructed using PhyloDraw (Choi *et al.*, 2000). Protein sequences were: AtABCC1 (At1g30400), AtABCC2 (At2g34660), AtABCC3 (At3g13080), AtABCC4 (At2g47800), AtABCC5 (At1g04120), AtABCC6 (At3g21250), AtABCC7 (At3g13100), AtABCC8 (At3g13090), AtABCC9 (At3g60160), AtABCC10 (At3g62700), AtABCC11 (At2g07680), AtABCC12 (At1g30420), AtABCC13 (At1g30410), AtABCC14 (At3g59140), AtABCC15 (At3g60970); OsABCC1 (Os04g52900), OsABCC2 (Os01g67580), OsABCC3 (Os01g07870), OsABCC4 (Os02g18700), OsABCC5 (Os02g18670), OsABCC6 (Os04g49890), OsABCC7 (Os04g49900), OsABCC8 (Os01g25386), OsABCC9 (Os04g13210), OsABCC10 (Os04g13220), OsABCC11 (Os06g36650), OsABCC12 (Os06g08560), OsABCC13 (Os03g04920), OsABCC14 (Os05g10730), OsABCC15 (Os06g06440), OsABCC16 (Os11g05700), OsABCC17 (Os12g37580); *Zea mays* ABCC1 (ZmABCC1; AAO72316), ZmABCC2 (AAO72318), ZmABCC3 (AAT37905); TaABCC2 (AAQ10074). Numbering scheme for AtABCCs and OsABCCs are defined by The Arabidopsis Information Resource (http://www.arabidopsis.org) and Klein *et al.* (2006), respectively. Nomenclature is adopted from Verrier *et al.* (2008).

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3. The Role of TtABCC3 in the Tolerance of Yeast to Cadmium

3.1. Introduction

The ATP-binding cassette subfamily C (ABCC) are integral membrane proteins found in a wide range of eukaryotes, from single-celled yeast to plants and humans (Holland *et al.*, 2003). All ABCCs contain two transmembrane domains (TMDs) and two cytosolic nucleotide-binding folds (NBFs) in a TMD-NBF-TMD-NBF arrangement (Verrier *et al.*, 2008). Each NBF contains the ABC signature sequence (Higgins *et al.*, 1986; Hyde *et al.*, 1990) flanked by the Walker A and B motifs (Walker *et al.*, 1982). Active transport of substrates by ABCs is energized by catalysis of ATP at the interface between the NBF domains (Linton and Higgins, 2007). The majority of ABCCs also have a hydrophobic N-terminal extension (NTE) domain which precedes the first TMD (Tusnády *et al.*, 1997). Some members lack this feature, including the *Arabidopsis thaliana* AtABCC13 (Kolukisaoglu *et al.*, 2002) and *Oryza sativa* OsABCC12 (Klein *et al.*, 2006), but are classified as ABCCs because of protein sequence homology with other members of the subfamily. The function of the NTE is poorly understood in plants, but studies on the human ABCC1 and the yeast cadmium factor 1 (YCF1) suggests the NTE is necessary for membrane trafficking, but not function (Mason and Michaelis, 2002; Westlake *et al.*, 2005).

In the budding yeast, *Saccharomyces cerevisiae*, the vacuolar pump YCF1 serves as the primary mechanism for cadmium (Cd) tolerance. YCF1 mediates the transport of bis(glutathionato)cadmium (GS_2-Cd) into the vacuole (Li *et al.*, 1996; Li *et al.*, 1997), and is also involved in sequestration of arsenic (Ghosh *et al.*, 1999) and mercury (Gueldry *et al.*, 2003). Strains that are devoid of YCF1 activity are hypersensitive to Cd

(Szczypka *et al.*, 1994). *YCF1* was the only gene identified in a screen of transformants from a yeast genomic library that permitted growth on plates containing 500 μM $CdCl_2$ (Szczypka *et al.*, 1994). Expression of *YCF1* is induced by Cd (Sharma *et al.*, 2002) and regulated by the transcription factor YAP1 (Wemmie *et al.*, 1994), which modulates the expression of numerous genes involved in oxidative stress (including Cd exposure). Of the other five ABCCs in yeast, only the bile pigment transporter 1 (BPT1) is implicated in Cd detoxification. BPT1 is the closest homologue to YCF1, is localized at the tonoplast, and mediates transport of glutathione-conjugated bile acids (Petrovic *et al.*, 2000). Although *bpt1* mutants have equivalent tolerance to Cd when compared to wild-type, *ycf1 bpt1* double mutants have greater Cd sensitivity than the *ycf1* mutant alone, suggesting BPT1 plays a minor role in Cd detoxification (Sharma *et al.*, 2002).

Heterologous expression of plant ABCCs in the Cd-hypersensitive *ycf1* mutant can be performed to identify those that are involved in Cd tolerance. Moreover, tonoplast-enriched vesicles isolated from these transformed strains can be used for kinetic studies of glutathione-conjugate transport because they have minimal endogenous glutathione-conjugate activity (Li *et al.*, 1996). *AtABCC3*, *4*, and *7* can partially rescue Cd tolerance in strains devoid of YCF1 activity (Tommasini *et al.*, 1998; Klein *et al.*, 2006); however, evidence for the transport of GS_2-Cd remains to be shown. Analysis of gene expression in seedlings of Arabidopsis exposed to 50 μM $CdCl_2$ for 24 h showed *AtABCC3* was induced in the roots but not in the leaves (Bovet *et al.*, 2003). Subsequent experiments using an Arabidopsis ABC-specific cDNA microarray found that Cd-induced expression of *AtABCC3* in roots was stronger than all other ABC genes (Bovet *et al.*, 2005). These results suggest AtABCC3 may play a role in detoxification of Cd in the

roots, but there is no published data for sensitivity of *atabcc3* mutant plants to Cd. Gaillard *et al.* (2008) found the expression of *AtABCC6* and *AtABCC7* in the roots is also elevated by Cd, but to a lesser extent than *AtABCC3*. These authors also proposed that the tandem organization of *AtABCC3*, *AtABCC6*, and *AtABCC7* on chromosome 3 might indicate a Cd detoxification cluster that responds to Cd toxicity. Whether the gene promoters have a common response element is unknown.

The TtABCC3 transporter is the most recent member of the ABCC subfamily to be identified in plants (Chapter 2). Two non-homeologous genes were isolated, designated α and β, which encoded transporters of 1496 and 1500 amino acids, respectively. Microarray expression analysis of root tissue from low-Cd and high-Cd accumulating near-isogenic lines (NILs) demonstrated that the expression of *TtABCC3* was higher in the low-Cd isoline (Appendix A). Greater quantities of TtABCC3 could decrease the cytosolic pool of Cd by sequestering Cd into the vacuole of root cells and limiting root-to-shoot Cd translocation. The hypothesis that an ABCC transporter could sequester Cd by transporting GS_2-Cd into the vacuole is also consistent with the concentrations of glutathione and phytochelatins likely to be present in the cytosol of plants grown on agricultural soils. Phytochelatins accumulate in the cytosol in response to toxic Cd concentrations (Cobbett and Goldsbrough, 2002), while glutathione is thought to be the predominant chelator ligand at non-phytotoxic concentrations of Cd (Wagner, 1993). Under these conditions, Cd^{2+} likely forms GS_2-Cd complexes instead of phytochelatin-Cd complexes. Given that *TtABCC3* was identified by microarray analysis of durum wheat seedlings grown in Cd concentrations typical of agricultural soils, TtABCC3 is an ideal candidate for GS_2-Cd transport. To test this hypothesis, the

TtABCC3 α and β variants were expressed in the Cd-hypersensitive *ycf1* mutant to see if Cd tolerance is restored, and evaluate if TtABCC3 is involved in Cd sequestration.

3.2. Materials and Methods

3.2.1. *S. cerevisiae* Strains and Growth Conditions

Isogenic yeast strains BY4741 (MAT*a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and Cd-hypersensitive *ycf1* mutant (MAT*a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 YCF1::kanMX4*) were obtained from the American Type Culture Collection (http://www.atcc.org; Manassas, VA, USA). Strains were grown for 3 d at 30°C on yeast peptone dextrose (YPD) plates [1% (w/v) bacto-yeast extract, 2% (w/v) bacto-peptone, 2% (w/v) glucose, 2% (w/v) bacto-agar] with or without 200 mg mL^{-1} geneticin to select for mutant strains.

The sensitivity of the *ycf1* mutant strain to Cd was confirmed by liquid and plate experiments. Yeast cultures of BY4741 and *ycf1* were grown aerobically overnight at 30°C (20 h, 225 rpm) in YPD medium, and washed twice in sterile YPD medium. For liquid assays, washed cells were used to inoculate 5 mL YPD medium containing 0, 30, or 60 μM $CdCl_2$ at an OD_{600} of 0.1. Growth curves were constructed from OD_{600} readings of 200 μL sub-samples measured in 96-well cell culture plates (Corning, Lowell, MA, USA) on a μQuant Microplate Spectrophotometer (BioTek, Winooski, VT, USA). For plate assays, washed cells were resuspended to an OD_{600} of 0.1, and 5 μL aliquots of several 10-fold dilutions were spotted onto plates containing 0, 25, 50, or 100 μM $CdCl_2$.

3.2.2. Transformation of *S. cerevisiae* with *TtABCC3α/β*

TtABCC3α/β inserts were obtained from pGEM-TtABCC3 plasmids (see Chapter 2) by digesting 2 µg of each plasmid with 20 U XhoI (GE Heatlhcare, Baie d'Urfé, QC, Canada) for 2 h at 37°C. Inserts were then separated on 0.8% agarose gel, excised, purified using QIAquick Gel Extraction kit (QIAGEN, Mississauga, ON, Canada), and subcloned into the *Escherichia coli* / *S. cerevisiae* multi-copy shuttle vector p423GPD (Figure 3.1; Mumberg *et al.*, 1995). The yeast glyceraldhyde-3-phosphate dehydrogenase promoter in p423GPD drives constitutive expression of inserts. To reduce self-ligation, the XhoI-digested p423GPD was incubated with 1 U Thermosensitive Alkaline Phosphatase (GIBCO-BRL, Grand Island, NY, USA) at 65°C for 30 min, followed by inactivation with 0.5 mM ethylenediaminetetraacetic acid (EDTA) for 15 min. Ligation was performed overnight (~20 h) at 4°C using an insert : vector ratio of 3 : 1. The ligated products were transformed into DH5α cells (Invitrogen, Burlington, ON, Canada) according to the manufacturer's protocol, and cells were grown overnight on plates containing 100 mg L^{-1} ampicillin. Successful transformants were identified by colony PCR with primers that spanned the insert-vector junction. Plasmids were purified using QIAprep Spin Miniprep kit (QIAGEN) and the presence of *TtABCC3α/β* inserts were confirmed by restriction digestion and sequencing.

Yeast strains BY4741 and *ycf1* were independently transformed with p423GPD or p423GPD harboring *TtABCC3α* (pTtABCC3α) or *TtABCC3β* (pTtABCC3β) using the high-efficiency lithium acetate/single-stranded carrier DNA/polyethylene glycol method (Gietz and Schiestl, 2007). Successful transformants were selected on synthetic complete (SC) plates without histidine (SC-HIS; Adams *et al.*, 1998). Preliminary experiments

showed the standard amino acid composition of SC (Rose *et al.*, 1990) contained insufficient leucine (30 mg L^{-1}) for normal growth, since growth was restored on plates with 100 mg L^{-1} leucine. Çakar *et al.* (1999) also demonstrated standard leucine concentrations of SC (30 mg L^{-1}) negatively affected the growth of leucine-auxotrophic yeast, such as those with *leu2* mutations.

3.2.3. Expression of *TtABCC3α/β* and *YCF1*

Total RNA was extracted from approximately 2×10^7 cells (culture OD_{600} = 2) from all six transformed yeast strains using the RNeasy Mini kit (QIAGEN, Mississauga, ON, Canada) with on-column DNase I treatment. Yeast cultures were grown overnight at 30°C (20 h, 225 rpm) in SC-HIS medium (Cd-free) to select for cells containing p423GPD constructs. RNA samples were quantified on a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Reverse transcription-polymerase chain reaction (RT-PCR) reactions were performed with the OneStep RT-PCR kit (QIAGEN) using 100 ng total RNA, 20 U RNaseOUT (Invitrogen, Burlington, ON, Canada), 1×Q-solution and 0.6 μM of each primer (Table 3.1). Cycling conditions were 50°C/30m, 95°C/15m, 25×(94°C/30s, 55°C/30s, 72°C/1m), 72°C/5m. The *TtABCC3* sense primers were specific for each variant. Primers for *TtABCC3β* targeted the 12 bp insert found only in *TtABCC3β*, whereas the *TtABCC3α* primer spanned this insert region. The endogenous *18S rRNA/RDN18* (SGDID S000006483; Table 3.1) was used as the RNA loading control.

3.2.4. Complementation Assays

Yeast cultures were grown aerobically overnight at 30°C (20 h, 225 rpm) in SC-HIS medium, and washed twice in sterile SC-HIS medium. Washed cells were used to inoculate 6 mL SC-HIS medium containing 0 or 50 μM $CdCl_2$ at an OD_{600} of 0.1. Growth curves were constructed from OD_{600} readings of 200 μL sub-samples measured at 4 h intervals for 72 h in 96-well cell culture plates (Corning, Lowell, MA, USA) on a μQuant Microplate Spectrophotometer (BioTek, Winooski, VT, USA). Experiments were performed in triplicate and samples were measured in technical triplicate.

3.2.5. Induction of *TtABCC3* by Cadmium

A pair of durum wheat NILs that differ in grain Cd accumulation (8982-SF-L/H, Low/High; Clarke *et al.*, 1997), were used in these experiments. Seeds were surface sterilized in 1.2% NaOCl for 20 min, rinsed in reverse osmosis (RO) water (<3 μS cm^{-1}), and imbibed for 36 h in an aerated solution of 1 mM $CaCl_2$ and 5 mg L^{-1} Vitavax fungicide (Uniroyal Chemical Ltd, Calgary, AB, Canada). Germinated seeds were placed on nylon mesh suspended over 10 L of aerated nutrient solution (Taylor, 1989). This nutrient solution was prepared in RO water and contained 1.0 mM $Ca(NO_3)_2$, 0.3 mM $Mg(NO_3)_2$, 0.3 mM NH_4NO_3, 0.4 mM KNO_3, 0.1 mM K_2HPO_4, 0.1 mM K_2SO_4, 2 μM $MnCl_2$, 6 μM H_3BO_3, 0.1 μM Na_2MoO_4, 0.5 μM $ZnSO_4$, 0.15 μM $CuSO_4$, 10 μM $FeCl_3$, 10 μM Na_2EDTA, 1 mM KOH, and 2 mM 2-(N-morpholino)ethanesulfonic (MES) acid buffer (pH 6.0). GEOCHEM-PC (Parker *et al.*, 1995) was used to calculate free ion/ligand/buffer activities (Table 3.2).

Seedlings were grown for 3 d in the dark, and then a further 4 d in a controlled environment growth chamber with day/night temperatures of 21/16°C (16/8 h), and a photon flux density of 450 μmol m^{-2} s^{-1}. The caryopses were removed after 7 d and seedlings were transferred to 10 L polyethylene buckets (under the same growth conditions) containing aerated nutrient solution as described above. Each bucket held two seedlings, supported independently by a polyethylene mesh basket mounted in opaque polycarbonate lids. Eight buckets were prepared for each SF-L and SF-H isoline, totalling 16 buckets with 32 seedlings. All buckets were suspended in a common water bath to maintain a consistent root temperature and limit temperature fluctuations between buckets. Throughout the growth period, solution pH and electrical conductivity (EC) were monitored daily, and RO water was added to maintain constant solution volume. If pH deviated from 6.0±0.1, solutions were adjusted with 1.25 N HNO_3 or KOH. Electrical conductivity was used to estimate nutrient solution depletion. Equal volumes of two daily addition stock solutions (Stock 1: 40 mM KH_2PO_4, 210 mM NH_4NO_3, 420 mM KNO_4, 20 mM $(NH_4)_2SO_4$; Stock 2: 20 mM $Ca(NO_3)_2$, 40 mM $Mg(NO_3)_2$, 0.4 mM H_3BO_3) were added to adjust the solution EC to the nominal level (575 μS cm^{-1}).

Seedlings were screened for a marker linked to the genetic inheritance of Cd accumulation (Penner *et al.*, 1995). Genotypes that possess this marker do not inherit the low cadmium uptake dominant gene *Cdu-1*. A single fragment is produced for high-Cd genotypes (*cdu-1*) and is absent for low-Cd genotypes (*Cdu-1*). The coleoptile was removed from each seedling prior to addition of $CdCl_2$ (see below), and shipped to C.J. Pozniak's laboratory for analysis (University of Saskatchewan, SK). Every seedling matched for marker screening and genotype of the plant.

To induce a response to Cd toxicity, 10 μM $CdCl_2$ was added to half of the buckets of each isoline 3 d prior to harvest. The free activity of Cd^{2+} was 3.93 μM (Table 3.2), which is 4-orders of magnitude greater than the free activity of Cd^{2+} in the chelator-buffered system (1.44×10^{-5} μM) used for the microarray experiments (see Chapter 2). Plants were harvested 14 d after being transferred to buckets (21-d-old plants). Roots were triple rinsed in RO water and the seedlings were divided into shoot and root tissues and blotted dry. Root tissue was immediately flash-frozen and stored at -80°C. The youngest fully-expanded lamina of the shoots was flash-frozen for RNA extraction, and the remaining tissue was oven-dried at 65°C overnight for analysis of Cd.

Shoot samples were digested in a 5 mL : 2 mL mixture of trace-metal grade concentrated HNO_3 and 30% H_2O_2, diluted to 50 mL with deionized water (>18 MΩ purity). The Cd concentrations of shoot tissues from untreated and Cd-treated seedlings were determined by graphite furnace atomic absorption spectroscopy (GF-AAS) or flame atomic absorption spectroscopy, respectively, on an AAnalyst 700 (PerkinElmer, Waltham, MA, USA). A National Institute of Standards and Technology (NIST) reference material (NIST no. 8436, durum wheat flour) was included in each batch of GF-ASS samples for quality control. Recovery of the certified Cd concentration (0.11±0.05) was 97±2% (mean±SD).

Total RNA was extracted from 50 mg of frozen, ground root and shoot tissue using the RNeasy Mini kit (QIAGEN, Mississauga, ON, Canada) and on-column DNase I treatment. RNA Samples were quantified on a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). RT-PCR reactions were performed with the OneStep RT-PCR kit (QIAGEN) using 250 ng total RNA, 20 U RNaseOUT (Invitrogen, Burlington, ON,

Canada), and 0.6 μM of each primer (Table 3.1). Cycling conditions were 50°C/30m, 95°C/15m, 30×(94°C/30s, 57°C/30s, 72°C/1m), 72°C/5m. The endogenous *β-Actin* (GenBank no. AB181991) was used as a RNA loading control.

3.3. Results

3.3.1. Heterologous Expression of *TtABCC3* in *ycf1* Mutant

YCF1 confers Cd resistance in yeast and disruption of this gene leads to Cd-hypersensitivity and cells devoid of GS_2-Cd transport across the tonoplast (Szczypka *et al.*, 1994; Li *et al.*, 1996; Li *et al.*, 1997). I confirmed the sensitivity of the *ycf1* mutant to Cd using both liquid and plate assays (Figure 3.2). Inhibition of growth was more pronounced in the *ycf1* mutant with increasing Cd concentrations when compared to the reference strain BY4741 (which has a functional *YCF1* gene). Exposure to 60 μM $CdCl_2$ retarded growth of *ycf1* by roughly 90% in liquid cultures when compared to untreated cultures (Figure 3.2A). In the plate assay, almost no growth was observed for the *ycf1* mutant at 50 μM $CdCl_2$, while BY4741 had a slight decease in growth (Figure 3.2B). There was no difference in growth between strains in the absence of Cd.

Suppressing the sensitivity of the *ycf1* mutant to Cd by heterologous gene expression infers that the gene product is involved in Cd tolerance. Several plant genes encoding putative ABCC transporters have been expressed in the *ycf1* strain to investigate Cd tolerance (and transport) including *AtABCC1* (Lu *et al.*, 1997), *AtABCC2* (Lu *et al.*, 1998), *AtABCC3* (Tommasini *et al.*, 1998), and *AtABCC5* (Gaedeke *et al.*, 2001). To assess whether TtABCC3 is involved in Cd tolerance, both *TtABCC3α* and *TtABCC3β* were expressed in *ycf1*. Expression of *TtABCC3α* and *TtABCC3β* was

confirmed using primer pairs that were specific for each variant (Figure 3.3). The *YCF1* transcript was detected in BY4741, but not *ycf1* mutants. Exposure to 50 μM $CdCl_2$ retarded growth in all strains (Figure 3.4), and the initiation of the logarithmic growth phase was delayed by nearly 20 h in the mutant. Neither *TtABCC3* variant restored Cd tolerance of the *ycf1* mutant, nor did they affect the growth rates of the reference strain BY4741 (Figure 3.4). The null vector p423GPD was also transformed into both strains, but had no affect on Cd tolerance. No difference in the growth was observed between strains when grown in the absence of Cd.

3.3.2. Induction of *TtABCC3* Expression by Cadmium

To examine whether expression of *TtABCC3* is induced by Cd toxicity *in planta*, RNA was extracted from SF-L/H seedlings that were exposed to 10 μM $CdCl_2$ for 3 d. The mean Cd content of shoots for 21-d-old SF-L and SF-H was 78.1±1.8 mg kg^{-1} (mean±SE; n = 8) and 75.3±4.0 mg kg^{-1}, respectively. The Cd concentration in shoots of plants treated with Cd was 3-orders of magnitude greater than in the untreated controls, which ranged from 6.1×10^{-3} mg Cd kg^{-1} to 1.9×10^{-2} mg Cd kg^{-1}. The biomass of the roots and shoots did not differ significantly between treatments. Root biomass from Cd-treated seedlings was 3.50±0.23 g (mean±SE; n = 8) and 3.36±0.30 g for SF-L and SF-H isolines, respectively, and 3.87±0.27 g and 3.36±0.12 g from untreated controls. The mean fresh weight of SF-L and SF-H seedling shoots was 3.08±0.23 g and 2.57±0.08 g for untreated and 3.02±0.16 g and 2.19±0.18 g for Cd-treated, respectively. Physical symptoms of toxicity were observed in shoots of plants treated with Cd. Leaves showed signs of wilting after 3-d exposure to 10 μM Cd, a symptom commonly associated with

Cd toxicity (Barcelò and Poschenrieder, 1990; Perfus-Barbeoch *et al.*, 2002). *TtABCC3* expression was detected in roots and shoots for both the SF-L/H NILs and exposure to 10 μM Cd did not alter expression levels (Figure 3.5).

3.4. Discussion

I hypothesized that TtABCC3 sequesters Cd into the vacuole of root cells of durum wheat, reducing the pool of available Cd for xylem-loading and root-to-shoot translocation. The complementation assays showed that neither *TtABCC3α* nor *TtABCC3β* restored Cd tolerance in the yeast *ycf1* mutant strain (Figure 3.4), suggesting that neither variant is involved in Cd tolerance in durum wheat. There are several possible explanations for these results: i) Incomplete translation of the *TtABCC3* mRNA; ii) Improper localization of the TtABCC3 protein; iii) TtABCC3 is not functional in yeast; iv) TtABCC3 is unable to transport GS_2-Cd; or iv) TtABCC3 can transport GS_2-Cd but not enough to compensate for the mutant phenotype.

Incomplete translation of the TtABCC3 protein could perhaps explain the inability of *TtABCC3* to restore Cd tolerance when expressed in the *ycf1* mutant. Expression of *TtABCC3α* and *TtABCC3β* was confirmed in the yeast transformants (Figure 3.3), but limited time did not permit experiments to be carried out to demonstrate that the full-length TtABCC3α/β proteins were present. Gaillard *et al.* (2008) reported that the *AtABCC6* sequence developed systematic mutations when expressed in yeast, resulting in a truncated protein. These authors attributed these mutations to AtABCC6 eliciting a toxic-response in the host. Expression of *AtABCC6* did not rescue Cd tolerance in the *ycf1* mutant, likely because the complete and functional protein was not present

(Gaillard *et al.*, 2008). Expression of *TtABCC3* could result in a similar toxic effect in yeast, resulting in a truncated, non-functional protein. Attempts to visualize TtABCC3 from crude total protein extracts from each transformed yeast strains were uninformative. These results indicate a more specific technique is required to identify TtABCC3 among the protein extracts. This could be accomplished by attaching an epitope tag to TtABCC3, such as FLAG (Hopp *et al.*, 1988) or c-*myc* (Evan *et al.*, 1985), and raising antibodies against the epitope on a Western blot to indicate the molecular weight of TtABCC3.

Heterologous expression of foreign proteins in yeast can lead to the formation of inclusion bodies, or insoluble aggregates of inactive proteins (Sambrook and Russell, 2001). Inclusion bodies can form when proteins are misfolded and internal hydrophobic residues remain exposed. Expression of the allilin lyase from garlic (*Allium sativum*) in *E. coli* and *S. cerevisiae* resulted in the formation of inclusion bodies in cytosol (Weik *et al.*, 1998). Misfolded proteins can also trigger proteolytic degradation by yeast proteases, which can be alleviated by using protease-deficient strains (Jones, 1991).

An alternative reason for the failure of *TtABCC3* to complement the *ycf1* mutant is that TtABCC3 may have been targeted to the incorrect membrane or no membrane at all. Although TtABCC3α and TtABCC3β are both predicted to localize to the plasma membrane rather than to the tonoplast (Chapter 2), they could still enable Cd tolerance by exporting Cd from the cytoplasm. AtABCC4 partially restores Cd tolerance in *ycf1*-defective strains and resides in the plasma membrane, indicating that tonoplast localization is not required (Klein *et al.*, 2006). The inaccuracies of computational prediction discussed in Chapter 2 demonstrate that membrane localization needs to be

established *in vivo*. Localization of TtABCC3 can be visualized in yeast by expressing a protein fusion construct of TtABCC3 with the green fluorescent protein (GFP; Tatchell and Robinson, 2002). Through the use of GFP-tagging, AtABCC1 and AtABCC4 were shown to localize to the tonoplast and plasma membrane, respectively (Geisler *et al.*, 2004; Klein *et al.*, 2004). In addition to the use of GFP protein fusions to determine subcellular localization, the GFP moiety can also be probed in Western blots with a GFP antiserum to provide information on protein expression and size.

The failure for *TtABCC3* to complement the *ycf1* mutant may be because the protein cannot bind/transport GS_2-Cd. Other plant ABCCs, such as AtABCC1, 2 and 5, also failed to rescue Cd tolerance in *ycf1*-defective strains (Lu *et al.*, 1997; Lu *et al.*, 1998; Gaedeke *et al.*, 2001). Transport experiments on vesicles isolated from these *AtABCC*-expressing strains demonstrated these proteins could transport other substrates, including the reduced and oxidized forms of glutathione, dinitrobenzene glutathione, and the chlorophyll catabolite *Bn*-NCC1. Therefore, TtABCC3 may mediate the transport of other substrates and function as a transporter in other processes in the plant.

Exposure of durum wheat seedlings to toxic concentrations of Cd showed that *TtABCC3* expression was not induced (Figure 3.5), suggesting that *TtABCC3* does not play a role in Cd detoxification. The ABCC transporters AtABCC1, 2, and 5 are also not induced by Cd stress (Bovet *et al.*, 2003). These transporters were also unable to complement the *ycf1* mutant and suggest gene expression and protein function could be coordinated for Cd tolerance. Plant growth in the experiments reported here did show symptoms of Cd toxicity after 3 d exposure, indicating that experimental conditions were indeed phytotoxic. There were, however, no significant differences in the biomass of

roots and shoots between treatments, which might be expected given that the length of exposure was only 3 d for 21-d-old plants. My analysis of gene expression made use of RNA from whole root/shoot samples. It is possible that expression of *TtABCC3* might be up-regulated in specific regions of the root/shoot, such as the root tip. Since sampling from whole tissues averages expression of *TtABCC3* from all regions, such localized expression might go undetected. Experiments on how other metals (such as zinc) or compounds (such as herbicide safeners) modulate expression of *TtABCC3* in durum wheat could also provide insight on the function of TtABCC3. Expression of *TaABCC1* is elevated under exposure to the herbicide safeners cloquintocet-mexyl and phenobarbital, suggesting that TaABBC1 may be involved in metabolism of herbicides (Theodoulou *et al.*, 2003).

Establishing the function of the TtABCC3 transporter will require more research. Experiments that elucidate the substrate specificity and subcellular localization of TtABCC3 in yeast should be informative for identifying the role of TtABCC3 in durum wheat. Moreover, TtABCC3 needs to be examined using plant systems to assess if the function of TtABCC3 relates to Cd accumulation.

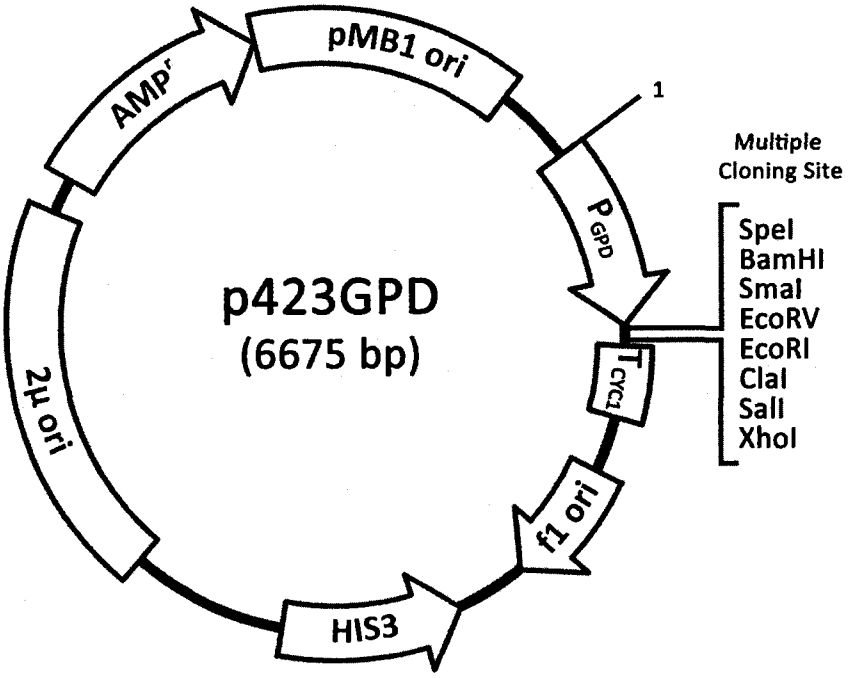
Table 3.1. Sequences and properties for primers used for RT-PCR.

Primer[1]	T_m (°C)	%GC	Complementary Score[2]		Sequence (5'→3')
			Any	3'	
Yeast RNA Expression					
TtABCC3α[Ins]-S	61.0	52.4	5	3	TACCTCGACAACAGCGGG
TtABCC3β[Ins]-S	60.8	61.1	4	0	TACCTTGACAACAGTGGCACC
TtABCC3[Ins]-AS	59.6	57.1	2	0	GACGGAGAGGAGAAGGAAGAG
YCF1-S	58.8	35.0	5	2	TTGGCTTTTTGAAACATTCG
YCF1-AS	60.1	36.0	3	0	AAGTTTGATGTATGTGTTGATGTGG
18SrRNA-S	60.0	50.0	4	0	ATGGCCGTTCTTAGTTGGTG
18SrRNA-S	60.0	50.0	4	0	ATGGCCGTTCTTAGTTGGTG
Cd Induction					
TtABCC3-S	59.8	55.0	4	1	AGAGCTGCAAAGGCTGGTAG
TtABCC3-AS	60.2	45.0	3	2	TATCCAAGCGGAAACAAAGC
Actin-S	59.9	52.4	3	4	GGTGAAGGGGACTTACAAAGG
Actin-AS	60.0	50.0	2	0	TTCATACAGCAGGCAAGCAC

[1] Primers were designed using Primer3Plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi; Rozen and Skaletsky, 2000).
[2] Local alignment score for self-complementation based on any allowable annealing (Any) or 3'-anchored annealing (3'). Scoring system: +1 Complementary; -1 Mismatch; -2 Gap. Lower is better.

Table 3.2. Total concentration, free activity and free concentration of ions, ligands, and buffers of the nutrient solution. Chemical speciation was calculated using GEOCHEM-PC.

	No Cadmium Added			Cadmium Added		
Chemical Species	**Total Concentration (μM)**	**Free Activity (μM)**	**Free Concentration (μM)**	**Total Concentration (μM)**	**Free Activity (μM)**	**Free Concentration (μM)**
Ca^{2+}	1000	709	982	1000	709	982
Mg^{2+}	300	213	295	300	213	295
K^{+}	1800	1660	1800	1800	1660	1800
Na^{+}	20.2	18.6	20.2	20.2	18.6	20.2
Fe^{3+}	10	1.77×10^{-9}	3.69×10^{-9}	10	1.85×10^{-9}	3.86×10^{-9}
Mn^{2+}	2	1.42	1.96	2	1.42	1.96
Cu^{2+}	0.15	3.53×10^{-4}	4.90×10^{-4}	0.15	6.70×10^{-4}	9.29×10^{-4}
Cd^{2+}	—	—	—	10	3.93	5.45
Zn^{2+}	0.5	0.140	0.194	0.5	0.195	0.271
SO_4^{2+}	101	63.8	88.4	101	63.8	88.4
Cl^{-}	34	31.3	34.0	54	49.7	54.0
NH_3	300	0.277	0.276	300	0.277	0.276
PO_4^{3-}	100	2.26×10^{-6}	4.71×10^{-6}	100	2.16×10^{-6}	4.50×10^{-6}
$B(OH)_4^{-}$	6	3.78×10^{-3}	4.10×10^{-3}	6	3.78×10^{-3}	4.10×10^{-3}
MoO_4^{2-}	0.1	0.071	0.099	0.1	0.071	0.099
NO_3^{-}	3300	3040	3300	3300	3040	3300
MES	2000	553	600	2000	553	600
EDTA	10	9.71×10^{-13}	3.58×10^{-12}	10	5.09×10^{-13}	1.88×10^{-12}



GPD promoter	1-649
Multiple cloning site	661-724
CYC1 transcription terminator	724-975
Phage f1 origin of replication	1178-1634
HIS3 gene	1894-2553 (complimentary strand)
2μ origin of replication	3121-4465
Ampicillin resistance gene	4598-5455
pMB1 origin of replication	5458-6398 (complimentary strand)

Figure 3.1. Schematic map of the *E. coli/S. cerevisiae* p423GPD shuttle vector.
Coding regions of genes and origins of replication are illustrated with arrows and blocks, respectively. Phage f1 origin of replication is drawn as an arrow to indicate direction of RNA synthesis. GPD, glyceraldehyde-3-phosphate dehydrogenase; CYC1, cytochrome c isoform 1; HIS3, imidazoleglycerol-phosphate dehydratase. Adapted from Mumberg *et al.* (1995).

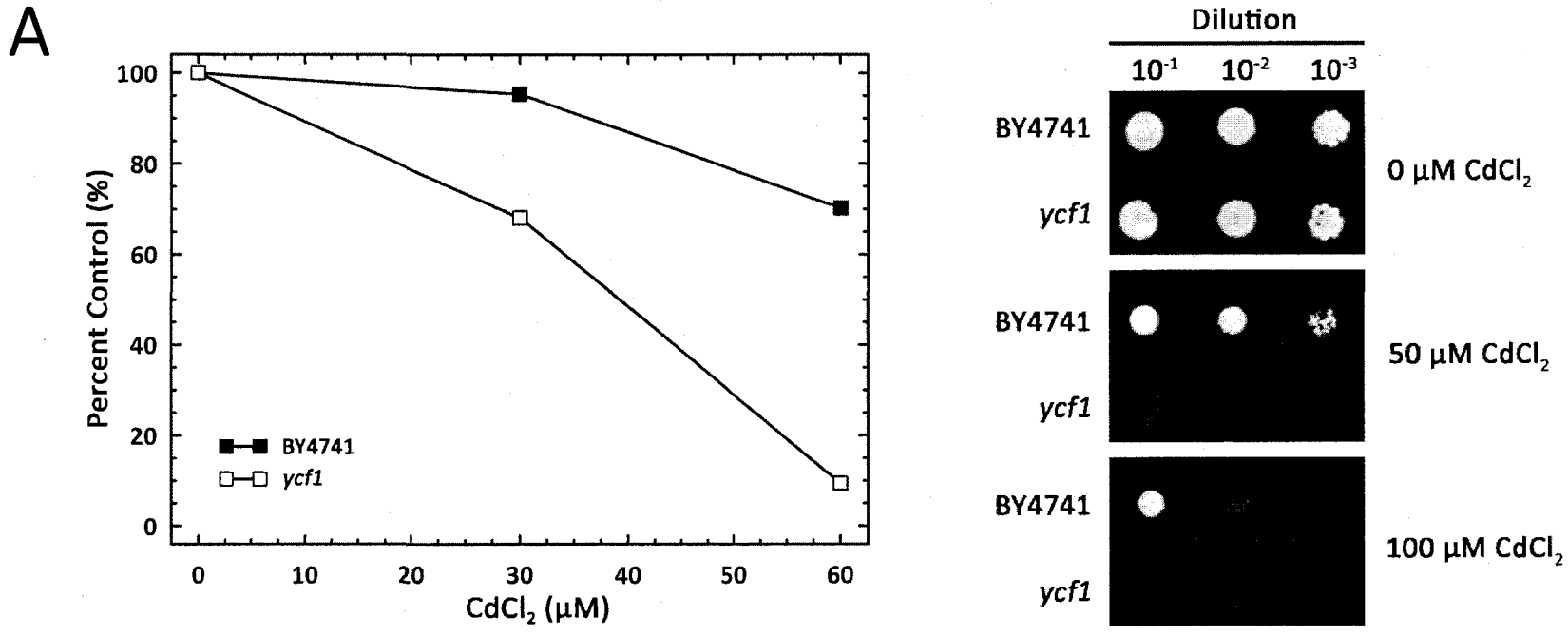


Figure 3.2. Confirmation of the sensitivity of the *ycf1* yeast mutant to cadmium.
(A) Liquid culture. Yeast cultures were grown at 30°C in YPD medium containing 0, 30, or 60 µM $CdCl_2$. Percent control values were calculated from OD_{600} measurements compared to untreated cultures at the 29 h time point. Data points represent an average percent (n = 3; ±SE). **(B) Plate assay.** Diluted cultures from a culture stock at OD_{600} = 0.1 were spotted (5 µL) onto YPD plates containing 0, 50, or 100 µM $CdCl_2$ and plates were grown for 3 d at 30°C. Spots represent one set of triplicate plating.

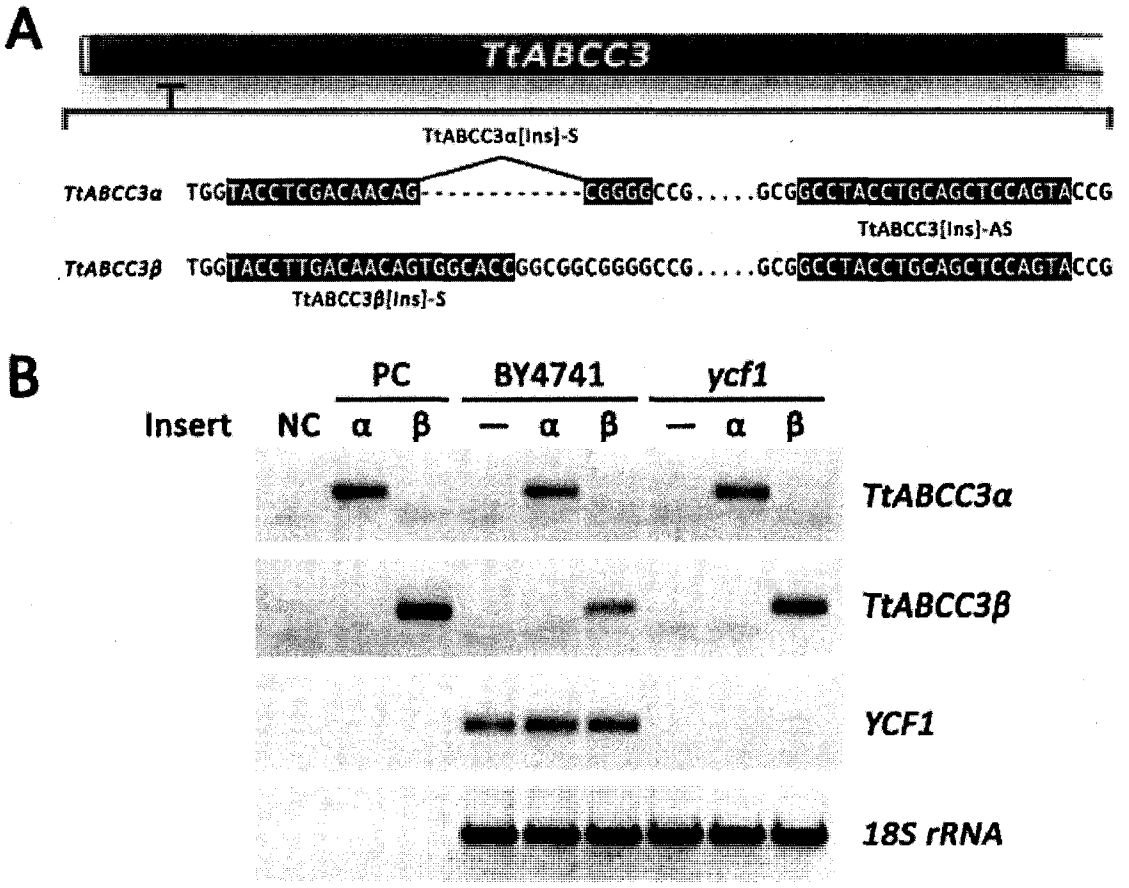


Figure 3.3. Expression of *TtABCC3* and *YCF1* in yeast transformants.
(A) Schematic of *TtABCC3* for α- and β-specific primer location. *TtABCC3* is shown with coding region (**black**) and untranslated regions (**white**). Primer sites are highlighted by **black** boxes. The TtABCC3α sense primer spans the 12 bp insert found in TtABCC3β, as indicated with dashes (- - -), and these primer sets were used for RT-PCR experiments. **(B) RT-PCR.** Total RNA was amplified from the yeast strains BY4741 and *ycf1* transformed with p423GPD (—), pTtABCC3α (α) or pTtABCC3β (β). Positive controls were purified plasmids used for transformation. *18S rRNA* was used as a loading control. NC, negative control (no RNA); PC, positive control.

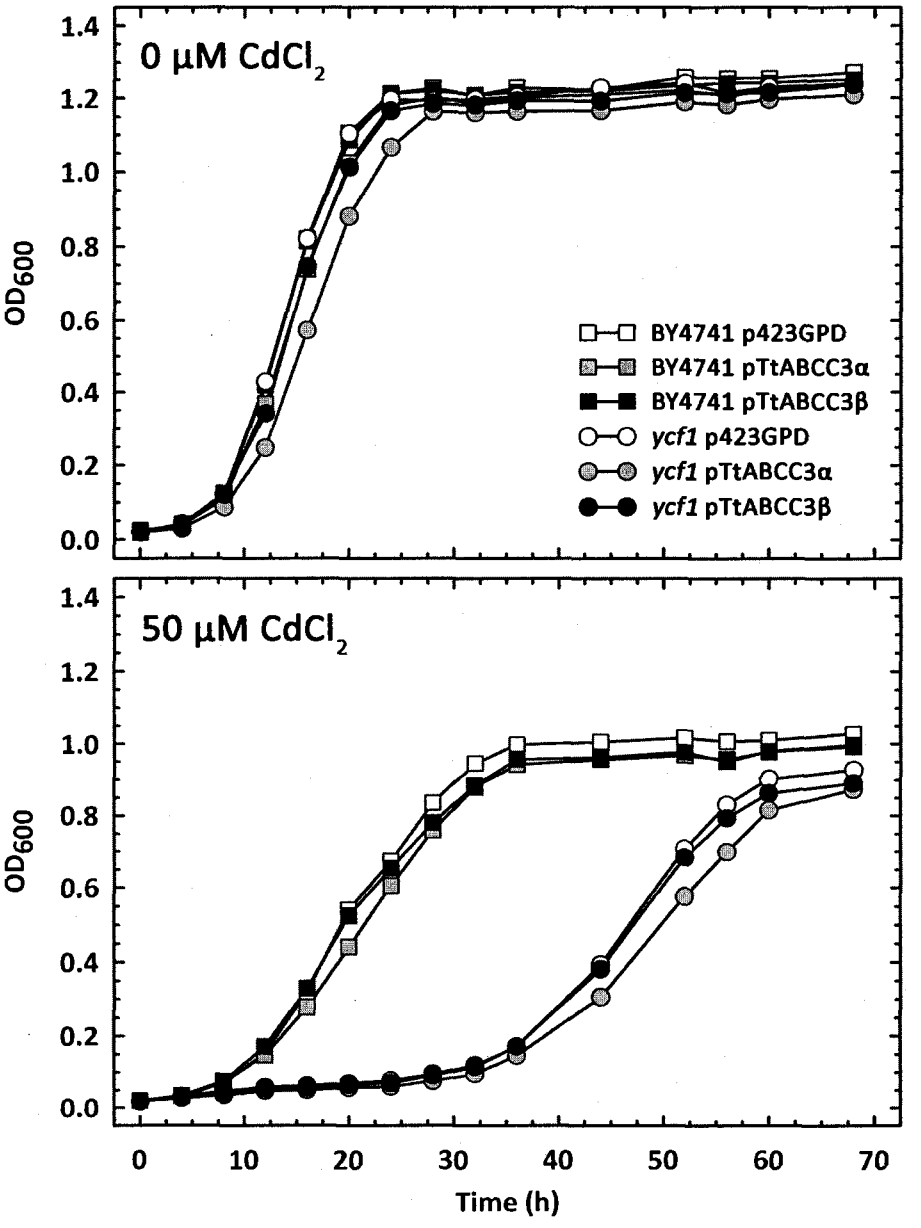


Figure 3.4. Complementation assays of *TtABCC3* in *ycf1* mutant.
Growth curves of the reference strain BY4741 (squares) and Cd-hypersensitive *ycf1* mutant (circles) transformed with empty p423GPD vector (p423GPD), p423GPD harboring *TtABCC3α* (pTtABCC3α) or *TtABCC3β* (pTtABCC3β). Cultures were grown at 30°C in SC-HIS medium containing 0 or 50 µM $CdCl_2$ and optical density was measured at 600 nm (OD_{600}) in 4 or 8 h intervals. Experiments were performed in triplicate and a representative is shown.

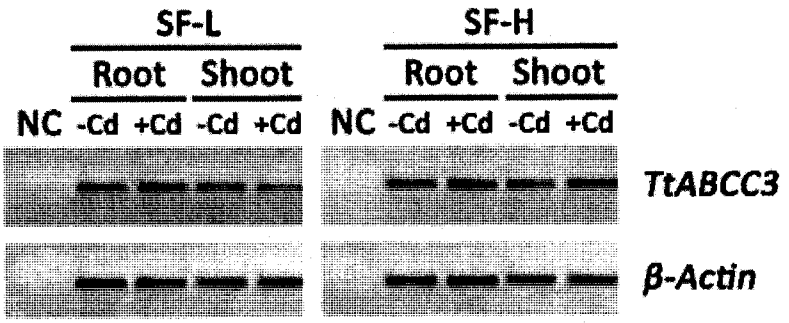


Figure 3.5. Effect of cadmium toxicity on expression of *TtABCC3* in roots and shoots of SF-L/H. Total RNA was extracted from root and shoot tissues of 3-week-old SF-L/H seedlings grown in the absence (-Cd) or presence (+Cd) of 10 μM $CdCl_2$ for 3 d. *TtABCC3* and *β-Actin* (RNA loading control) were amplified using RT-PCR. NC, negative control.

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4. Conclusion

Cadmium (Cd) accumulates in the grain of durum wheat (*Triticum turgidum* L. var. *durum*) and consumption of this grain is a dietary source of Cd for humans. The potential health concerns associated with consumption of Cd-containing foodstuffs have prompted the development of low grain-Cd durum cultivars to reduce Cd intake in the human diet (Grant *et al.*, 2008). Differences in accumulation of Cd in the grain have been primarily attributed to the retention of Cd in the roots of low-Cd cultivars. Reduced translocation of Cd may be caused by increased vacuolar sequestration and/or reduced xylem loading (Harris and Taylor, 2004). However, our understanding of the underlying molecular mechanism for root retention still remains unknown.

Microarray analysis of root tissue from low-Cd and high-Cd accumulating near-isogenic lines (NILs) provided an initial list of differentially expressed genes that could be involved in regulating Cd accumulation in the grain of durum wheat (Appendix A). One of these genes encoded a *T. turgidum* ATP-binding cassette subfamily C 3 (TtABCC3) transporter. I hypothesized that TtABCC3 would increase sequestration of Cd in the vacuole of root cells, reducing the pool of Cd available for xylem loading and root-to-shoot translocation of Cd. To test this hypothesis, the full-length coding region of *TtABCC3* was isolated and expressed in a Cd-hypersensitive yeast strain — which lacks the ABCC transporter yeast cadmium factor 1 (YCF1) — to see if Cd tolerance could be restored.

The full-length coding sequence of *TtABCC3* was obtained using expressed sequence tag (EST) resources, combined with rolling circle amplification - rapid amplification of cDNA ends (Chapter 2). *TtABCC3* is the third wheat ABCC to be

characterized (after *TaABCC1* and *TaABCC2* from *T. aestivum*), and only the second full-length *Triticum* sequence to be isolated (partial mRNA exists for *TaABCC1*; Theodoulou *et al.*, 2003). Sequencing of *TtABCC3* clones identified two non-homeologous genes, *TtABCC3α* and *TtABCC3β*, which corresponded to the A and B genomes of the tetraploid durum wheat, respectively (C.J. Pozniak, University of Saskatchewan, personal communication). These genes are the first polyploid set of ABCCs to be isolated, suggesting that (non-)homoelogous gene sets likely exist for other wheat ABCCs. Heterologous expression of *TtABCC3* in the *ycf1* mutant strain is also the first functional characterization of an ABCC transporter from *Triticum* species to be documented. Current literature on wheat ABCCs has only reported the induction of *TaABCC1* by xenobiotics (Theodoulou *et al.*, 2003).

The failure of *TtABCC3α* or *TtABCC3β* to complement the Cd-hypersensitive *ycf1* mutant suggests that neither TtABCC3 variant plays a role in Cd tolerance (Chapter 3). Moreover, *TtABCC3* expression was not induced following 3-d exposure to 10 μM Cd *in planta*. Under the assumption that Cd is sequestered into the vacuole by similar mechanisms at toxic and non-phytotoxic concentrations, the proposed function of TtABCC3 as a transporter of glutathione-Cd conjugates into the vacuole is not supported. Additional experiments that demonstrate TtABCC3 is entirely translated and localized to the tonoplast in yeast are required before the hypothesis can be completely refuted. The results from the yeast complementation experiments also question whether TtABCC3 is even relevant to the regulation of Cd accumulation in grain of durum wheat, and why *TtABCC3* was differentially expressed between the NILs. It is possible that *TtABCC3* co-segregated with another gene associated with reduced translocation of Cd to the grain,

such that both were selected during development of the isolines. However, the mapping of *TtABCC3α* to chromosome 3A indicates that this gene is not located at the loci, which is on chromosome 5B (Knox *et al.*, 2003). Differential expression of *TtABCC3* between the low/high NILs could also arise from different rates of mRNA transcription due to variations in the nucleotide sequence of the promoter region. Potential differences in the nucleotide sequence can be investigated by isolating and sequencing the promoter regions for both isolines. The promoters of *TtABCC3α* and *TtABCC3β* within each isoline could also be compared to infer if expressional differences occur between variants. Alternatively, the gene responsible for the genetic inheritance of Cd accumulation may encode a transcription factor that modulates the expression of several genes, including those (such as *TtABCC3*) that may not affect the root-to-shoot translocation of Cd in durum wheat.

Microarray analysis demonstrated that several genes are differentially expressed between low-Cd and high-Cd NILs (Appendix A). Although I choose to investigate *TtABCC3*, other potential gene candidates still remain. One of these genes encoded a putative glutamate decarboxylase [annotated as GAD1 (*Hordeum vulgare*); Table A.1] and was more highly expressed in the high-Cd isoline. GAD is a cytosolic enzyme that catalyzes the conversion of glutamate to c-aminobutyrate (GABA) in the GABA-shunt metabolic pathway (Bouché *et al.*, 2003). Up-regulation of *GAD* in the high-Cd isoline could diminish the glutamate pool in the cytosol, and since glutamate is a precursor for glutathione (GSH) synthesis, this could reduce the amount of cytosolic GSH (Mendoza-Cózatl *et al.*, 2005). Since GSH is involved in the chelation of Cd and the synthesis of

phytochelatins, reduced levels of GSH may increase the available Cd pool for root-to-shoot translocation.

The *Triticum* microarrays used to identify candidate genes involved in Cd accumulation in grain of durum wheat do have several limitations, including the probe-set coverage of the transcriptome and the probe design. Although the probe set was designed from a database of *T. aestivum* ESTs enriched in abiotic stress-responsive transcripts (Houde *et al.*, 2006), the microarray represents only a subset of genes from the wheat genome. In the absence of a sequenced genome for *Triticum* species, the number of genes in the wheat genome is unknown, but likely resembles the diploid rice, which has over 56,000 genes identified (TIGR rice genome annotation, release 5, January 2007; http://rice.plantbiology.msu.edu). Thus, the 17,300 gene-specific probe set is only a fraction of the approximately 50,000 genes in each A, B, and D genome of *T. aestivum*. This concern of genomic under-representation may be alleviated by using the recent commercial microarray Affymetrix GeneChip Wheat Genome Array (http://www.affymetrix.com/products/arrays/specific/wheat.affx), which has over 61,000-probes representing over 55,000 unique transcripts. The use a polyploid species for probe design can also lead to probe redundancy, as was observed for the two spots that were differentially expressed and hybridized to different regions of *TtABCC3*. Redundancy of probes also results from incomplete gene coverage by ESTs, especially genes with large coding regions. Sometimes when ESTs are assembled they can generate sequence clusters along the gene that are disconnected from each other, and probes are designed from these different clusters.

The microarray approach assumes differences between NILs are due to differing gene expression, but these differences could also result from protein regulation. Differences in protein activity between the isolines could be due to differing post-translational modifications. These modifications could alter protein function that leads to the retention of Cd in the root system of the low-Cd isoline. Protein activity could also be affected by point mutations in the coding region of the gene without altering gene expression. For instance, these mutations could cause a frameshift or non-sense mutation that results in a premature stop codon in the mRNA transcript and forms a non-functional, truncated protein. Therefore, proteomic analysis of the NILs may be a complementary approach to the microarray analysis for identifying genes (and their gene products) potentially involved in Cd accumulation. Microarray analysis is also not useful if the gene responsible for the grain Cd accumulation trait is not expressed in one of the low/high isoline pairs, since the raw fluorescence data of the corresponding spot would be below a threshold level and would be omitted from statistical analysis.

The role of TtABCC3 in durum wheat still remains unknown despite *TtABCC3* being isolated and functionally characterized in *Saccharomyces cerevisiae*. These results showed that neither *TtABCC3* variant can restore Cd tolerance in the Cd-hypersensitive *ycf1* strain nor is *TtABCC3* induced by Cd stress in durum wheat seedlings. The following experiments should help in deciphering the role of TtABCC3 (both α/β variants) in durum wheat:

- Creating protein fusions between the green fluorescent protein and TtABCC3 to examine the subcellular localization of TtABCC3 in yeast and (if possible) durum wheat. Protein fusions of AtABCC1 and AtABCC4 showed these

transporters are localized to the tonoplast and plasma membrane, respectively (Geisler *et al.*, 2004; Klein *et al.*, 2004).

- Using membrane vesicles isolated from *ycf1* strains of yeast expressing *TtABCC3* to analyze the substrate specificities and kinetic properties of TtABCC3. Vesicles isolated from *AtABCC*-expressing yeast transformants were used to investigate substrate and kinetic properties of Arabidopsis ABCC transporters (Klein *et al.*, 2006).
- Combining the *TtABCC3* promoter sequence with a reporter gene, such as β-glucuronidase (GUS), to visualize the expression pattern of *TtABCC3* in durum wheat. Expression of *AtABCC6::GUS* constructs in Arabidopsis showed *AtABCC6* is mainly expressed in germinated seeds and seedlings (Gaillard *et al.*, 2008).
- Assaying the expression levels of promoter/reporter gene constructs, such as GUS or luciferase, to examine promoter activity at basal condition and when exposed to various stresses and/or chemicals. The *Zea mays ZmABCC3* promoter was fused with the luciferase gene to show expression of *ZmABCC3* is regulated by anthocyanin (Goodman *et al.*, 2004).

The experimental approach used here to isolate and characterize *TtABCC3* also provides a framework for studying other genes that are identified from microarray analysis, such as *GAD1*. Following the isolation of the full-length coding region of *GAD1*, the gene would be expressed in the yeast *gad1* mutant. These mutants are sensitive to oxidants, including hydrogen peroxide (Coleman *et al.*, 2001), but would

need to be characterized for Cd-related phenotypes (Cd tolerance, and accumulation) before attempting complementation with *T. turgidum GAD1*.

Ongoing fine mapping of the low grain-Cd locus on chromosome 5B has also identified a set of genes that could be involved in the accumulation of Cd in grain of durum wheat (C.J. Pozniak, University of Saskatchewan, personal communication). Using a collection of deletion lines of *T. aestivum*, ESTs are being mapped to chromosomes, and then grouped into 'bins' that represent a region of a chromosome (Qi *et al.*, 2004). Bin16 of chromosome 5B includes the *Cdu1* locus responsible for the grain-Cd accumulation trait, but also has ESTs that match to *TaABCC1* and *GAD1*. *TaABCC1* can be isolated and characterized using the same approach as described for *TtABCC3*. This would involve isolating the remaining sequence of the full-length coding region of *TaABCC1* (as only a partial mRNA is currently available), and then expressing the full-length in *ycf1* to see if *TaABCC1* restores Cd tolerance.

Identification of the gene(s) involved in regulating accumulation of Cd in durum wheat grain will provide researchers and breeders with information to assist in the development of low-Cd cultivars. These cultivars will be specifically selected for the low grain-Cd accumulation trait, while preserving other desirable traits such as micronutrient content and grain yield. Furthermore, identification of the these gene(s) will likely increase our understanding of the genetic mechanism of Cd accumulation in other crops that accumulate Cd, such as rice, flax and potato (McLaughlin *et al.*, 1999). If orthologous genes were found in those crops, this would accelerate the development of low-Cd cultivars.

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Appendix A. Microarray Analysis of Near-Isogenic Lines of Durum Wheat that Differ in Accumulation of Cadmium in Grain[2]

A.1. Materials and Methods

A.1.1. Microarray Preparation and Hybridization

The microarrays consisted of a 19.2 k spotted array (with 17.3 k gene-specific), printed 'in-house' on UltraGAPS coated slides (Corning, Lowell, MA, USA). Annotated *Triticum aestivum* expressed sequence tags (ESTs) from the Genome Canada Functional Genomics of Abiotic Stress project (Houde *et al.*, 2006) were used for probe design. Given that the genome of *T. turgidum* ($2n = 4x = 28$; AABB genomes) represents a subset of the genome of *T. aestivum* ($2n = 6x = 42$; AABBDD genomes), the probe-set enables interspecific hybridization of *T. turgidum* transcripts to the spotted oligonucleotides. Probes were constructed as long oligonucleotides (70-mers) as described in Mohammadi *et al.* (2007), including quality control parameters for GC content, vector contamination, and melting temperature. Each spot was assigned a unique identification number (UID).

The microarrays were prepared and hybridized according to an unpublished Genome BC Forestry microarray protocol. Briefly, total RNA was extracted from 750 mg of frozen root tissue using the RNeasy Maxi kit (Qiagen, Mississauga, ON, Canada), which included on-column DNase I treatment. Labeled cDNA was generated by incorporating Cy3- or Cy5-dUTP in combination with 60 μg total RNA, 25 ng μL^{-1} oligo(dT) primer, 40 U RNaseOUT (Invitrogen, Burlington, ON, Canada), and 200 U Superscript II (Invitrogen). Reactions were incubated for 2.5 h at 42°C, followed by RNA

[2]The microarray experiments and data analysis were completed prior to the start of this graduate program. The microarray results (N.S. Harris and G.J. Taylor, unpublished) are relevant to this research and are included for completeness.

hydrolysis in 0.167 mM NaOH for 15 min at 65°C, and neutralization in 0.167 mM HCl/Tris-HCl (pH 7.5). Labeled cDNAs were purified using QIAquick PCR purification kit (Qiagen), and paired Cy3-labeled and Cy5-labeled samples combined and ethanol precipitated at -20°C overnight. To reduce dye incorporation bias, a dye-flip approach was applied to half of the replicates for each isoline pair (TL pools 2 and 4; SF pools 3 and 4). Prior to hybridization, cDNAs were denatured at 95°C for 5 min and maintained at 65°C in SlideHyb Glass Array Hybridization Buffer #1 (Ambion, Austin, TX, USA).

The arrays were rehydrated prior to hybridization, UV cross-linked (1200 mJ for 5 min), washed in 1% sodium dodecyl sulfate (SDS) for 5 min, ethanol-rinsed, and spun-dry. Arrays were incubated in pre-hybridization solution (5X SSC [0.75 M NaCl, 0.75 M trisodium citrate], 0.1% SDS, 0.2% bovine serum albumin) for 60 min at 48°C. For hybridization, denatured probes were aliquoted onto each slide, a Lifterslip coverslip (Fisher Scientific, Pittsburgh, PA, USA) was applied, and slides incubated 16 h at 42°C. The coverslips were removed by rinsing in pre-warmed (42°C) 2X SSC, 0.5% SDS solution. The slides were washed in the same solution for 15 min at 42°C, washed twice in 0.5X SSC, 0.5% SDS for 15 min at 42°C, rinsed in 0.1X SSC at room temperature, and dried by centrifugation at 1000 × *g*. Slides were scanned on arrayWoRx^{e} reader (Applied Precision, Issaquah, WA, USA) using excitation wavelength of 540 and 635 nm for Cy3 and Cy5, respectively.

A.1.2. Data Analysis

The Institute for Genomic Research (TIGR) microarray analysis suite (TM4, http://www.tm4.org; Saeed *et al.*, 2003) was used to for data analysis. Raw fluorescent

data were extracted with Spotfinder v3.1.1, using the Otsu threshold algorithm (Liao *et al.*, 2001) for spot identification. Quality control (QC) filters included background (BKG) correction, keep flagged values, and QC threshold of [1 × BKG median + 2 × BKG standard deviation]. The raw data were normalized by LOWESS block normalization, followed by standard deviation block regularization settings using MIDAS v2.1.9. The normalization data for both isolines (SF and TL) were combined and analyzed using MeV v4.0. Spots with no integrated spot intensity were excluded and the remaining 10221 spots were subjected to significance analysis of microarrays (SAM) analysis (Tusher *et al.*, 2001). Differentially expressed genes were selected using a ≤5% false discovery rate (FDR) cutoff (Table A.1).

Annotations of the differentially expressed genes were updated by MEGABLAST search of the 70-mers against the Dana-Farber Cancer Institute (DFCI) *T. aestivum* gene index (*Ta*GI, release 10.0; http://compbio.dfci.harvard.edu/tgi) and the TIGR Plant Transcript Assemblies (PTA) for *T. aestivum* release 2, *T. monococcum* release 2, and *T. turgidum* release 1 (http://plantta.tigr.org; Childs *et al.*, 2007). In cases where the 70-mer annotations were different between the databases, *Ta*GI was used over PTA.

A.2. Results and Discussion

SAM analysis of two pairs of low- and high-Cd accumulating near-isogenic lines (NILs), TL and SF, identified 29 differentially expressed genes that could potentially be involved in regulation of Cd accumulation in durum wheat (Figure A.1). These genes included two desaturases, two germins (A and F), chitinase III, a ribosomal protein, and a

DNA-directed RNA polymerase. Seven spots encoded genes for hypothetical proteins or had no annotation assigned (Table A.1).

Of the 29 differentially expressed genes, one spot (UID10434) encoded a putative, multidrug resistance-associated protein (MRP) transporter. Yeast MRP, yeast cadmium factor 1, has been shown to sequester Cd into the vacuole via glutathione-mediated transport (Li *et al.*, 1996; Li *et al.*, 1997). Glutathione-conjugate Cd transport has also been suggested for MRP homologues that rescue Cd-hypersensitive *ycf1* mutants, including those from humans (Tommasini *et al.*, 1996), Arabidopsis (Tommasini *et al.*, 1998; Klein *et al.*, 2006), and the algae *Chlamydomonas reinhardtii* (Wang and Wu, 2006). Given that the putative MRP transporter showed higher expression in the low-Cd isoline, this protein could function in vacuolar sequestration of Cd, thereby lowering the available Cd for root-to-shoot translocation.

An additional spot (UID418) that also annotated as a putative MRP transporter was identified by the microarray, but with a FDR that was above the cutoff threshold (Figure A.1). Compared to UID10434, these spots had a similar differential expression patterns across all pools, suggesting that this MRP may also be involved in the regulation of Cd accumulation in durum wheat.

Both spots matched unique TCs, suggesting the microarray identified two putative MRP transporters from durum wheat. With these TCs as a starting point, additional sequence information can be obtained from EST datasets, such as *T. aestivum* dbEST from the National Center for Biotechnology Information. Once the full-length coding region is isolated, the gene products can be characterized for their putative function.

Table A.1. Annotation summary of microarray 70-mers found to be differently expressed between low-Cd and high-Cd NILs of durum wheat.

UID	70-mer Sequence (5' -> 3')	Tentative Annotation	Source[1]
5354	TTGTCCGGGAACTTCGGCAGGACTCTGCCAGCCAGC ATGGCCTCCTTCCTGGAGTAGTTACCAAGCTTAC	putative transmembrane protein [*Arabidopsis thaliana*]	TC263266
2243	CACAAAAACCGCCGATTTTTCACCCCAAGGACAGC GATTCCTCGTAGCTGTGGCGGTGGATCCATGGCTG	putative UVB-resistance protein UVR8 [*Oryza sativa* (japonica cultivar-group)]	TC238792
16997	TAATAGGGATGTGGCGTTTTTGTAGTTGTTCGAACC TGTCTCTCTGGGGTTGCTGGACGATAGCTTCCAG	unknown protein [*Arabidopsis thaliana*]	TC276304
13411	GCAGGAGGAGGAGAGAAGAGACACGCTTGTGACCT GTCGGTCTCCATCACTTTCTTGATTTGTGGCCTGA	putative auxin-induced protein IAA18 [*Oryza sativa* (japonica cultivar-group)]	TC270980
10950	TGGTTTGTTGCGAGTTCGAGCTGTGGTGACTGTATC GGCGTTGGGTTGAGTGTTTGCTATTTTTATTTTT	beta-glucosidase isozyme 2 precursor [*Oryza sativa* (japonica cultivar-group)]	TC268338
11101	GGGCAATTGGGTCTATAATTTGTAAGGTGTTCGATC CAATTGCGAACAGGAGGAGCTGGAGTACGCGGAG	putative NHL repeat-containing protein [*Oryza sativa* (japonica cultivar-group)]	TC245375
13384	GCCAACCTGTTGCTTCTTAACAGAACCAAAAAGGTT TGGACCTTGGCCTAGAAGTAGCTAGCAAGAGAGA	No annotation assigned	CV772601
13815	GCTGCCGAGTACGAGAAGAGACGGGAGAACTTCGT CAAAGAGATTGACATCGTCATCGCTGATGTGGTCA	hypothetical protein [*Oryza sativa* (japonica cultivar-group)]	TC240656
12523	TTCTGAATCATGTTTTGTAAAGGAAGTTAAAAGCCC ATGCGAGTGAGACAATGGTTGCGGTGTGGCTGAC	hypothetical protein [*Oryza sativa* (japonica cultivar-group)]	TC236991
190	CCCCAGCAGCACCTGAAGGCTATGTTGCCTATTCTA TTTAGTTGTAGCTGGCTTTGTGGATGTCTGTTAT	putative ribosomal protein L35A [*Oryza sativa* (japonica cultivar-group)]	TC263076
4830	ATTGCACTCTCCCTTTCGGCCCTGCTGGCGATTGGTT CTGCACAGGACACCTCTCCACCGAGGTCACTAT	GAD1 [*Hordeum vulgare*]	TC247291
13494	ACGAGTACACAGCCGAGACTCCCCCACTCAAAATG AAGAAGGACTACTCCATGGTTCCAATAATAGGAAA	No annotation assigned	CV770265
10128	CCAGCCTCCTGGGATCTCGGATTGGTGAGTCCATGA ATGTACATCATGAAACACCAATGTACGTAGTAAG	fatty acid desaturase FAD7 [*Zea mays*]	TC248578
14932	GTCCATTTGGTACCATACTGCACACGGGGGACTGTC GGCTCACATCACATTGTGTGCAGAAATTGCTACT	DNA ligase-like [*Oryza sativa* (japonica cultivar-group)]	TA105983_4565
2874	GGATCCCCGGTCTCAACACACTGGGTATCTCCTTAG CACGCATTGACTATGCACCTTTGGGGGAGAACCC	germin-like protein 1 precursor [*Oryza sativa* (japonica cultivar-group)]	TC259407
3563	TTCTCTCTAGGGCATCTCCCCGCTCCGATCGATGGC GTCGTCCTGCATCCCGACGGGCATCCGGCTGCCG	putative CIL [*Oryza sativa* (japonica cultivar-group)]	CK197923

2607	GCTGGCAATGATTGGGGTTCTTGCAGGTCTGTCCTT TGTGATGGGGCCTCTTAAAATGCTAAAGCTCTAT	plastid omega-3 fatty acid desaturase [*Oryza sativa* (japonica cultivar-group)]	TA58108_4565
13165	CTGCAGTCCTCGTACCAGGTTTTGTTGCCTTCAAGC AAGCGAAATCCCAAAAGCCTCCGAGAATTGAACT	No annotation assigned	TC261339
9765	CGATCGTTCACTAGCTACACACACATCACGTAGTCA GGCTAAGCTAGGAAGAAGCACTCGCCCTCGGCGA	putative 60S ribosomal protein L37a [*Oryza sativa* (japonica cultivar-group)]	TA60181_4565
326	CCCCTTACAGCCATACCCAAGGAAATATGACTTGTA TTCTCCCGAGCGGCTCATTTCATTTCATCCTGTA	putative cytochrome P450 monooxygenase [*Oryza sativa* (japonica cultivar-group)]	CV774636
13043	AAGACCTCCTGGCTAGCACGCGTGTGCAGTTCAAGT CCATGGCACAGAAGGGGTTTCTGGCAGCCGAGAA	putative glucan 1-3-beta-glucosidase [*Oryza sativa* (japonica cultivar-group)]	TC235234
3303	GCATTGCCGACATGAAGGCGCCTGGTATGCACTATA CATATTATGTGCGCGTATACATGTGTGTCCTTAA	germin F [*Hordeum vulgare*]	CK199341
3687	GCTGCAATTCGGAATTGTAAAGGAAGCTATATAAG ACCGACGGACAAAATGTGTGCCAGGGTGCTAAACA	putative serine carboxypeptidase [*Oryza sativa* (japonica cultivar-group)]	TC256215
1754	AGTTAGCTAGCTCGACGCACGTAATTCTTCACACCA ACAAGAAAACTCATCTACCCACCAATGGCGCTCG	xylanase inhibitor protein 1 precursor [*Oryza sativa* (japonica cultivar-group)]	TC264928
6469	GCGGCGCACAAATAAGACAAGTTTGCTGCATACAG CATATATACGTACGAAGCCATGTATCCCGGATATG	putative class III chitinase [*Oryza sativa* (japonica cultivar-group)]	TC266128
2003	CGCACTAGTTTGCGGTTCATTAGGATCAGGATTTGC GATGTTATGACATTGACTCTGACTCGTGTTCCAG	GPX12Hv glutathione peroxidase-like protein [*Hordeum vulgare*]	CV776961
7432	GTTCAAGCAGACGGGTTGGATTAATCAGACCAAATT CACCCAAGTCCCCTTTTGCTAGTGCAAGTACTTT	hypothetical protein [*Oryza sativa* (japonica cultivar-group)]	BE604868
13101	GTCCCAGAGAAATCCTGATCGACGCTACTGCTTTGG CTTTCTTGTCCAAACGCCATCTACTTTATTGTAC	Metallo-beta-lactamase-like [*Oryza sativa* (japonica cultivar-group)]	TC248871
10434	GAGTGGCTTTGTTTCCGCTTGGATACGCCTGTCGTCC TTCACATTTGCATTCGCTTTGGTATTTCTGATC	putative MRP-like ABC transporter [*Oryza sativa* (japonica cultivar-group)]	TC258389
418	GACCCTCTTAACGAATACAACGACGATCAGATCTGG GAGTCCTTGGATAACTGTCAGCTGGGAGATGAGG	putative MRP-like ABC transporter [*Oryza sativa* (japonica cultivar-group)]	TC237754

[1]Annotations are acquired from transcript assemblies constructed by: TCXXXXXX, DFCI *T. aestivum* gene index release 10.0 (http://compbio.dfci.harvard.edu/tgi; Quackenbush *et al.*, 2001); TAXXXXX(X)_4565, TIGR Plant Transcript Assemblies for *T. aestivum* release 2, *T. monococcum* release 2, and *T. turgidum* release 1 (http://plantta.tigr.org; Childs *et al.*, 2007). All others represent singleton ESTs acquired from the DFCI *T. aestivum* gene index.

Mean log_2 ratios (H/L)	q-value (%)	TL pools 1 2 3 4	SF pools 1 2 3 4	UID	Annotation
0.26	3.44			5354	putative transmembrane protein [*Arabidopsis thaliana*]
0.50	3.44			2243	putative UVB-resistance protein UVR8 [*Oryza sativa* (japonica cultivar-group)]
0.43	3.44			16997	unknown protein [*Arabidopsis thaliana*]
0.26	4.64			13411	GH14349p [*Drosophila melanogaster*]
0.32	4.64			10950	beta-glucosidase isozyme 2 precursor [*Oryza sativa* (japonica cultivar-group)]
0.69	4.64			11101	putative NHL repeat-containing protein [*Oryza sativa* (japonica cultivar-group)]
0.41	4.64			13384	DNA-directed RNA polymerase (isoform B1)-like protein [*Oryza sativa* (japonica cultivar-group)]
0.32	0.00			13815	unknown protein [*Oryza sativa* (japonica cultivar-group)]
0.56	0.00			12523	PREDICTED P0048D08.116 gene product [*Oryza sativa* (japonica cultivar-group)]
0.35	0.00			190	putative ribosomal protein L35A [*Oryza sativa* (japonica cultivar-group)]
1.04	0.00			4830	GAD1 [*Hordeum vulgare*]
0.86	0.00			13494	unknown protein [*Xenopus laevis*]
-0.39	0.00			10128	chloroplast omega-3 desaturase [*Prunus persica*]
-0.35	0.00			14932	P0018C10.37 [*Oryza sativa* (japonica cultivar-group)]
-0.47	0.00			2874	putative germin A [*Oryza sativa* (japonica cultivar-group)]
-0.21	0.00			3563	putative CIL [*Oryza sativa* (japonica cultivar-group)]
-0.40	0.00			2607	omega-3 fatty acid desaturase FAD8 [*Zea mays*]
-0.55	0.00			13165	ENSANGP00000022061 [*Anopheles gambiae*]
-0.45	0.00			9765	putative lipase [*Oryza sativa* (japonica cultivar-group)]
-0.48	0.00			326	putative cytochrome P450 monooxygenase [*Oryza sativa* (japonica cultivar-group)]
-0.62	0.00			13043	putative Glucan 1;3-beta-glucosidase precursor [*Oryza sativa* (japonica cultivar-group)]
-0.48	0.00			3303	germin F [*Hordeum vulgare*]
-0.35	0.00			3687	putative serine carboxypeptidase [*Oryza sativa* (japonica cultivar-group)]
-0.28	4.22			1754	chitinase III C10701 [*Oryza sativa* (japonica cultivar-group)]
-0.27	4.22			6469	putative class III chitinase [*Oryza sativa* (japonica cultivar-group)]
-0.30	3.87			2003	glutathione peroxidase-like protein [*Hordeum vulgare*]
-0.40	3.87			7432	putative AMP deaminase [*Arabidopsis thaliana*]
-0.23	3.31			13101	PREDICTED OJ1124_D06.11-2 gene product [*Oryza sativa* (japonica cultivar-group)]
-0.31	3.20			10434	putative MRP-like ABC transporter [*Oryza sativa* (japonica cultivar-group)]
-0.21	18.56			418	putative MRP-like ABC transporter [*Oryza sativa* (japonica cultivar-group)]

Figure A.1. Differentially expressed genes identified by microarray analysis of low- and high-Cd NILs of TL and SF pools.
Red and green indicate expression levels that are lower or higher in the low-Cd isoline (L) when compared to the high-Cd isoline (H), respectively. UID, unique identification number.

A.3. Literature Cited

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