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Full Length Research Paper

Various mitochondrial endogenous kinase activities in fertile and cytoplasmic male sterile wheat (*T. aestivum*)

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The *orf256 gene* is associated with cytoplasmic male sterile (CMS) wheat. The gene lies upstream of *coxI* in *T. timopheevi* (Tt) mitochondrial DNA (mtDNA) and is expressed with *coxI* in the same mRNA in Tt, fertility restored (FR), and CMS lines. It is not present in *Triticum aestivum* (Ta) mtDNA. The gene is expressed as a 7 kD protein only in CMS lines. Different transcripts were detected in the different mitochondrial sources. Orf256-coxI RNA binds to many protein from various wheat mitochondrial sources. Here, we report that there were various kinase activities in mitochondrial protein extracts of Ta and CMS wheats. Three bands of mitochondrial proteins were subject to phosphorylation by endogenous kinase activity that was differentially modulated by sodium vanadate. Dephosphorylation of mitochondrial protein extracts seems to decrease the kinase activity or make those protein bands less favorable substrates for the endogenous kinases. Staurosporine does not have major effect on the kinase activities. Results showed that there might be different kinases, one of which is modulated by sodium vanadate but not with staurosporine, in various mitochondrial protein sources. The variations in kinase activity may reflect proteomic variations among mitochondrial protein sources.

Key words: wheat, mitochondria, protein, endogenous kinase activity, sodium vanadate, staurosporine, cytoplasmic male sterility (CMS).

INTRODUCTION

CMS trait is developed in alloplasmic wheat lines where nuclear DNA is introduced into cytoplasm from different species (Kihara, 1951) leading to nuclear cytoplasm incompatibility. The most common one is an alloplasmic line with *T. aestivum* (Ta) nucleus and *T. timopheevi* (Tt) cytoplasm which gives rise to completely sterile plants after recurrent backcrosses of F1 to Ta (Wilson and Ross, 1962).The alloplasmic nature of CMS led to the concept that CMS is produced as a result of nuclear-cytoplasmic incompatibility. This is due to differences in the nuclear genes when they are put in a different cytoplasm. Introduction of Ta nuclear genes into Tt cytoplasm during CMS construction could develop a nuclear-cytoplasmic incompatibility. When Tt nuclear genes are introduced during fertility restoration of CMS, they establish a sound nuclear-cytoplasmic interaction with Tt mitochondria and consequently fertility restoration. The open reading frame, *orf256*, is located upstream of *coxI* in fertile Tt, CMS, and FR, but not in Ta mitochondrial DNA (mtDNA) (Rathburn and Hedgcoth, 1991; Rathburn *et.* al, 1993). The upstream sequence from -228 to -1 and the first 33 nucleotides of the coding sequence of the *orf256* are identical to those of *coxI* of Ta, but the rest of the *orf256* sequence is not related to that of coxI (Rathburn and Hedgcoth, 1991). A protein of 7 kD was detected in mitochondrial proteins of CMS lines using antibodies raised against a predicted *orf256* peptide which was not detected in the mitochondrial proteins from Ta, Tt, or FR lines (Song and Hedgcoth, 1994). The *orf256* sequence was found in various species of wheat relatives and progenitors, but was expressed as RNA only in Tt and *Aegilops speltoides.* None of the tested plants have the expression of 7 kD

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protein except the CMS plants (Hedgcoth et al, 2002). Various mitochondrial proteins from CMS, Ta, FR, and Tt, bind to the o*rf256-coxI* RNA, but two proteins of about 42 and 39 kD proteins were detected only in Ta mitochondrial extract. These proteins could have been involved in elimination of the *orf256* sequence from Ta mtDNA (El-Shehawi et al, 2003). The affinity of mitochondrial proteins to *orf256-coxI* depends on their phosphorylation state, which is independent of the 5' or 3' UTR regions. Biotin-labeled *orf256-coxI* RNA was used for affinity purification of RNA binding proteins (El-Shehawi and Hedgcoth, 2004). RNA editing events in the 5' region of *orf256* do not directly explain transcriptional or translational differences for *orf256* gene expression, yet they indicate a possible relationship between the nuclear and mitochondrial genomes in mitochondrial functions in wheat (El-Shehawi et al, 2004).

Endogenous kinase activity is an effective assay that has been used for detection and isolation of kinases (Kann et al, 1993; SidAhmed-Mezi et al, 2010; Faustino et al, 2010). An endogenous kinase activity (probably of cellular origin) of the hepatitis B virus was characterized in the presence of different ions and inhibitors. This enzyme phosphorylates serine residue(s) localized within the lumen of the particle. The endogenous protein kinase of the core particles was not inhibited by staurosporine, a selective inhibitor of PKC. In contrast, quercetine, a selective inhibitor of the protein kinase M (PKM) inhibited the kinase activity during later phases of incubation (Kann et al, 1993). Endogenous kinase activity assay was used in the purification and determination of the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to be the kinase involved in the endogenous phosphorylation of the alpha1 subunit of the gamma-aminobutyric acid (GABA)(A) receptor. Accordingly, a method was developed to identify these atypical membrane-bound phosphatases (SidAhmed-Mezi et al, 2010). Endogenous kinase activity was identified within the nuclear pore complex (NPC) fraction, which phosphorylated a 30 kDa nuclear pore protein (Nup30). Enriched NPCs revealed constitutive presence of all members of the MAP kinase family, ERK, p38, and JNK. The NPC thus contains a spectrum of associated MAP kinases that suggests an intimate role for ERK and p38 in regulation of nuclear pore function (Faustino et al, 2010). In addition, endogenous kinase activity has been used for estimation of kinase activities (Van Loon et al, 1975; Ehrnsperger et al, 2001; Seo et al, 2003; Kann et al, 1991; Faustino et al, 2010; Lilienthal et al, 2010). Endogenous kinase activity was determined in the nuclei of sterile embryos of barley (*Hordeum vulgare*) and cultures of *Lemna perpusilla*. It was able to phosphorylates chromatin proteins with molecular weights ranging from 10 kD to 100 kD (Van Loon et al, 1991). Major vault protein (MVP) is known to be phosphorylated by protein kinase C in vitro and by tyrosine kinase in vivo. MVP from two mammalian cell lines (CHO and PC12 cell) was highly phosphorylated by endogenous protein kinases in cell-free systems. Phosphorylation of MVP inhibited by EDTA. In contrast to CHO cells, addition of recombinant casein kinase II enhances the phosphorylation of MVP in PC12 cells (Ehrnsperger et al, 200). A non-radioactive and highly sensitive enzyme-linked immunosorbent (ELISA)-based kinase assay was developed using the phosphospecific antibody in biological samples. The method was applied to measure the endogenous DYRK1A activity in mouse Sodium vanadate is an inhibitor of tyrosine phosphatase (Rybaczek et al, 2002; Watanabe et al, 2009). Sodium vanadate was capable to inhibit premature chromosome condensation (PCC) in root meristem cells, as well as the range of possible regulatory pathways leading to the transition from S-phase arrest towards abnormal mitosis (Rybaczek et al, 2002). It was shown to modulate the intracellular Cl^- concentrations and gamma-aminobutyric acid (GABA) responses in neonatal neurons by increasing the proportion of neuronal K^+ -Cl^- cotransporter (KCC2) associated with lipid rafts membrane domains. Loss of tyrosine phosphorylation also reduced oligomerization of KCC2 (Watanabe et al, 2009). In addition, sodium vanadate can inhibit other phosphatases (Hawker et al, 1987; Berdyshev et al, 2011). Sucrose phosphatase was reported to be more sensitive to inhibition by sodium vanadate and less sensitive to ammonium molybdate than was the nonspecific phosphatase in vacuole extracts of sugacane and red beet. Sucrose phosphatase was suggested to be part of the group translocator proposed to operate in the tonoplast of sugarcane and red beet (Hawker et al, 1987). Addition of sodium vanadate increased sphingosine-1-phosphate (S1P) by blocking phosphatases including lipid phosphate phosphatases (LPPs) (Berdyshev et al, 2011). Moreover, sodium vanadate can activate kinases (Huang et al, 1998; Papapetropoulos et al, 2004). Vanadate enhances the phosphorylation of IkappaBalpha which suggests the activation of AP-1 and NFkappaB. This could be mediated through enhancement of phosphorylation of Erk/p38 kinases and IkappaBalpha, respectively (Huang et al, 1998). Vanadate activates the phosphoinositide3-kinase (PI3-K)/Akt pathway, which is known to increase endothelial NOS (eNOS) activity by direct phosphorylation of Ser-1179. Treatment of BLMVEC with vanadate resulted in phosphorylation of both Akt and endothelial NOS. In addition, wortmannin, a PI3-K inhibitor, blocked both the vanadate-induced phosphorylation of eNOS and the increase in cGMP accumulation (Papapetropoulos et al, 2004).

The results of experiments of RNA-binding protein, RNA editing, expression of orf256 in *E. coli*, did not explain the molecular difference between CMS and other fertile wheat species, therefore, we used the endogenous kinase activity assay combined with phosphatase and /or kinase inhibitors (Sodium vanadate and Staurosporine) to

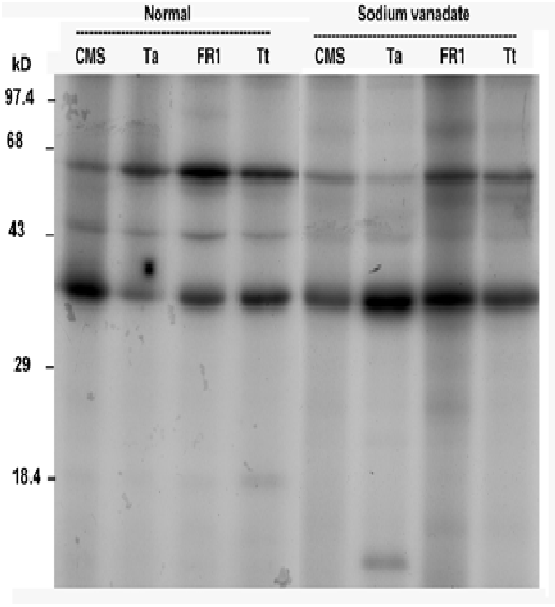

Figure1: Endogenous kinase activity of CMS, Ta, FR1, and Tt normal mitochondrial protein extracts compared to the same extracts prepared in the presence of sodium vanadate.

investigate possible differences in the endogenous kinase and/or phosphatase activity among wheat lines especially CMS and other fertile species (Ta, FR1, Tt).

MATERIALS AND METHODS

Preparation of Mitochondrial Proteins

The following wheat lines were used in this study: 1) *T. aestivum (*Ta) accession Newton; 2) CMS accession A910; 3) *T. timopneevi* (Tt) accession TA103; and fertility restored accession WH1020. Wheat lines were generously obtained from Wheat Genetic Resource Center at Kansas State University (http://www.k-state.edu/wgrc). Wheat shoots preparation and isolation of mitochondria was carried out according to El-Shehawi et al (2003). Etiolated wheat shoots (7-10 days old) were harvested with a sterile, cold scalpel in one inch segments onto aluminum foil chilled on a bed of ice. Shoots were weighed quickly and homogenized in a cold mortar using three volumes of ice-cold homogenization buffer per gram of shoots (10 mM TES, pH 7.2; 1 mM EDTA; 0.5 M manitol; 0.2% bovine serum albumin, fraction V, fatty acid free (Sigma); 0.02% L-cysteine (Sigma). All subsequent steps were done at 4°C. The homogenate was filtered through four layers of cheesecloth and one layer of miracloth, premoistened in homogenization buffer, into a cold beaker. The tissues were rehomogenized and filtered into the same beaker. The homogenate was centrifuged for 10 min at 1,000 g. The supernatant was centrifuged for 10 min at the same speed. The supernatant was centrifuged for 10 min at 12,000 g to pellet mitochondria. The supernatant was recentrifuged to pellet more mitochondria. Using a small, sterile, artist's brush, the mitochondrial pellet was gently suspended in 10 mL of homogenization buffer by lightly stroking the surface of the pellet. The mitochondrial suspension was centrifuged for 10 min at 1,000 g. The supernatant was carefully layered onto 10 mL of cushion buffer (10 mM TES, pH 7.2; 20 mM EDTA; 0.6 M sucrose) and centrifuged for 20 min at 10,000 g. The mitochondrial pellet was suspended in 10 mL of cushion buffer and centrifuged for 10 min at 12,000 g. Mitochondria were washed one more time in 10 mL of cushion buffer. The mitochondrial pellet was stored at - 20°C as one pellet or distributed in microfuge tubes (pellet from 2 to 3 g of shoots) at the last step. Microfuge tubes were centrifuged for 10 min at 12,000 g. The supernatant was removed and pellets were stored at -20°C. Mitochondrial protein extract was prepared by sonication according to a published procedure (Song and Hedgcoth, 1994; El-Shehawi et al, 2003). Bradford Reagent (Bio-Rad) was used to determine protein concentration at 595 nm using a bovine serum albumin standard curve.

Endogenous Kinase Activity and SDS-PAGE

Endogenous phosphorylation in mitochondrial extracts was done according to a published procedure (Vazquez-Tello et al, 1998; Harter et al, 1994). Mitochondrial protein extract, 20 µg, was incubated in kinase buffer (30 mM Tris-HCl, pH 7.0; 1 mM $CaCl_2$; 2 mM $MgCl_2$; 0.2 mM EDTA) containing 50 µCi of [γ-^{32}P]ATP for 30 min at room temperature. Proteins were separated by 12%SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The gel was dried for 1 hr at 80°C under vacuum and exposed to X-ray film overnight. Scanned photos of X-ray films were used to quantitatively estimate the possible differences in band intensities using TotalLab software.

SODIUM VANADATE TREATMENT

Sodium vanadate, 2 mM, was included in all buffers and solutions used for mitochondrial isolation and mitochondrial protein extracts preparation.

Dephosphorylation of Mitochondrial Protein Extracts

Mitochondrial protein extracts were treated with alkhaline phosphatase before they were used for endogenous kinase activity. Forty µg of mitochondrial protein extract were incubated with alkhaline phosphatase beads (Sigma) for 15 min. Alkhaline phosphatase beads were removed by centrifugation and protein concentration was estimated in the dephosphorylated protein samples.

Staurosporine Treatment of Mitochondrial Protein Extracts

Staurosporine, 10 µM, was added to mitochondrial protein extracts for 10 min before the addition of [γ-^{32}P] ATP.

RESULTS

To investigate if there is a kinase activity differences in various mitochondrial extracts under study, [γ-^{32}P] ATP was used as a substrate for endogenous kinases. Endogenous kinase activity showed phosphorylation of three major protein bands of 60, 46, and 35 kD (Fig.1, Fig.2, Fig.3). All bands are detected in all extracts. However, it is clear that there are some intensity variations among the different mitochondrial protein extracts. Generally, in the normal extracts the 60 kD band was weaker in CMS, stronger in Ta, FR1, and Tt. The 46 kD band showed the same intensity in all mitochondrial

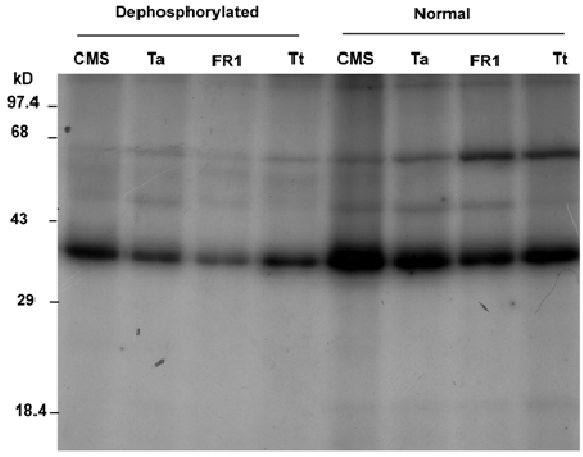

Figure2: Endogenous kinase activity of CMS, Ta, FR1, and Tt normal and dephosphorylated mitochondrial protein extracts.

Lane		Intensities		
		60 kD	46 kD	35 kD
Normal	CMS	36.35	18.22	100.39
	Ta	97.56	20.66	45.17
	FR1	146.62	41.63	89.24
	Tt	130.94	29.04	109.28
Sodium vanadate	CMS	45.19	14.82	85.40
	Ta	29.47	20.19	151.47
	FR1	71.27	11.28	113.23
	Tt	68.524	17.04	104.76

Table 1: Summary of densitometric analysis of detected bands in normal mitochondrial protein extracts CMS, Ta, FR1, Tt compared to the same extracts prepared in the presence of sodium vanadate.

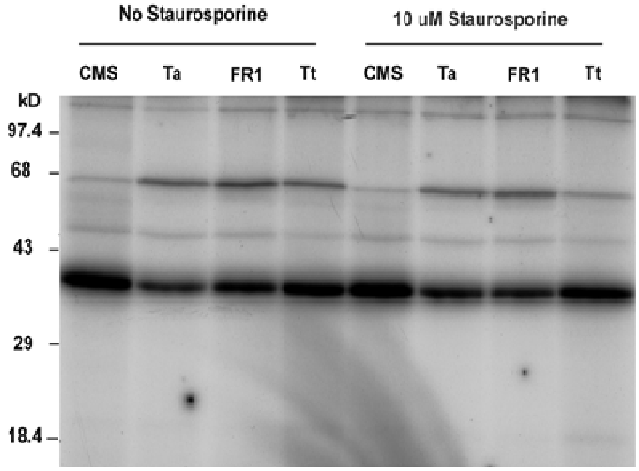

Figure3: Comparison of endogenous kinase activity of CMS, Ta, FR1, and Tt mitochondrial protein extracts in the presence and absence of staurosporine.

extracts. The 35 kD band showed highest intensity in CMS, lowest intensity in Ta and intermediate intensity in FR1 and Tt (Fig1- 3, Table1- 3). The 60 kD band showed higher intensity in Ta, FR, and Tt extracts, whereas it had lower intensity in the CMS extract. The 46 kD band had the same intensity in all extracts. Differences in band strength and subsequently kinase activity suggest that there is a difference in kinase activity, especially between CMS and other extracts. The presence of the 46 kD band with the same intensity in all extracts serves as a good internal control for equal protein loading. This does not roll out the possibility that the various intensity could be a result of differences in certain protein substrate for the kinase. Sodium vanadate is phosphatase inhibitor. Isolation of mitochondrial protein extracts in the presence of sodium vanadate prevents the removal of phosphates from protein during isolation of mitochondria and mitochondrial protein extracts preparation. Using mitochondrial extracts isolated in the presence of sodium vanadate for estimating the endogenous kinase activity showed that the 35 kD band was less intense in CMS and more intense in Ta (Fig.1). This could be due to the effect of sodium vanadate as a phosphatase inhibitor (Morioka et al, 1998; Wo et al, 1998) in keeping some proteins phosphorylated, like the 35 kD band in CMS, so that the endogenous kinases can not add much label to it. The 35 kD band intensity in Ta may be enhanced as a result of the effect of sodium vanadate as a kinase enhancer (Pandey et al, 1998; Pandey et al, 1999). The kinase responsible for phosphorylation of the 35 kD band may be activated by sodium vanadate or another hyperphosphorylated protein as a response to the presence of vanadate salt in Ta extract. Considering these possibilities, it can be concluded that the phosphorylation of the 60 kD and 35 kD bands may be attributed to the activity of two different kinases which are modulated differently by sodium vanadate at least in CMS and Ta extracts.

Quantitative analysis showed that the 60 kD band had stronger intensity in the presence of sodium vanadate in CMS compared to normal CMS mitochondrial protein extract (36.35 and 45.19 respectively) (Table 1). On the other hand, intensity was about three folds in Ta without sodium vanadate compared to its intensity in the presence of sodium vanadate (97.56 and 29.47 respectively). Also, its intensity was about two folds without sodium vanadate in FR1 (146.62 and 71.27) and Tt (130.94, 68.52) compared to their intensities in the presence of sodium vanadate (Table 1). Phosphatase inhibition seems to keep the 60 kD protein hyperphosphorylated which makes it less phosphorylated

Lane		Intensities		
		60 kD	46 kD	35 kD
Dephosphorylated	CMS	11.5	11.33	142.47
	Ta	12.44	12.53	104.58
	FR1	13.24	13.74	101.95
	Tt	14.47	13.53	120.08
Normal	CMS	25.19	17.56	350.14
	Ta	28.44	16.11	253.58
	FR1	40.89	14	149.5
	Tt	38.39	15.11	152.42

TABLE 2: Summary of densitometric analysis of detected bands in mitochondrial protein extracts CMS, Ta, FR1, Tt after dephosphorylation compared with normal extracts.

Lane		Intensities		
		60 kD	46 kD	35 kD
No staurosporine	CMS	28.78	12.35	195.73
	Ta	69.53	23.19	140.62
	FR1	118.78	24.20	162.98
	Tt	87.96	19.92	161.29
+ staurosporine	CMS	29.25	24.81	189.98
	Ta	88.51	23.16	162.02
	FR1	79.73	20.86	147.89
	Tt	45.38	12.36	180.44

TABLE 3: Summary of densitometric analysis of detected bands in mitochondrial protein extracts CMS, Ta, FR1, Tt in the presence or absence of staurosporine (kinase inhibitor).

by the kinase activity. Another scenario is that the inhibition of phosphatase reduces the kinase activity itself because it may need dephosphorylation for full activity. The 46 kD band showed little variations in intensities in all lines without sodium vanadate (18.22 to 41.63) or with sodium vanadate (20.19 to 11.23) (Table 1). The 35 kD band had higher intensity in the presence of sodium vanadate in Ta, FR1, and Tt (151.47, 113.23, 104.76 respectively), but weaker intensity in CMS (85.40) compared to their lower intensities in the absence of sodium vanadate (45.17 89.24 109.28) and higher intensity in CMS (100.39). This means that inhibition of phosphatases during isolation and preparation of mitochondrial extracts reduce the kinase activity in CMS whereas it enhances kinase activity in Ta, FR1, and Tt.

Estimation of endogenous kinase activity can be misleading to some extent since sometimes a kinase target is already phosphorylated, therefore it is important to remove phosphates from the target protein before estimation of the kinase activity. Dephosphorylated mitochondrial extracts were used for endogenous kinase activity along with normal extracts. Dephosphorylated extracts showed lower kinase activity in all lines compared to normal ones for both the 60 and 35 kD bands (Fig. 2). It is clear from Fig (2) and Table (2) that dephosphorylation decreased the intensity of all bands, but there is differential response among various mitochondrial extracts. The 60 kD band showed very close intensities in the dephosphorylated mitochondrial extracts and the normal extracts. The 46 band also showed very close intensities in the normal and dephosphorylated extracts in all mitochondrial sources (Fig. 2, Table 2). The 35 band showed lower intensity in CMS compared to the same normal extract, but higher intensity compared to other dephosphorylated extracts. CMS showed about to 2 folds in the normal state compared to the dephosphorylated CMS extract. Ta and FR1 showed three folds in the normal extract compared to the dephosphorylated Ta and FR1 respectively. Tt showed about 25% higher in the normal state compared to its intensity in the dephosphorylated state (Table 2).

Staurosporine is the most potent PKC inhibitor. In addition, it inhibits cAMP- and cGMP-dependent kinases and inhibits phosphatases. It was used in the endogenous kinase activity assay at 10 µM to determine the nature of the kinase which carries out the phosphorylation activity in the various mitochondrial extracts. The inhibitor was incubated with normal protein extracts for 10 min at room temperature before the addition of [γ-^{32}P]ATP. Staurosporine seems to reduce the phosphorylation of the 60 kD band only in Tt extract, whereas it does not reveal differences in the phosphorylation of the 35 kD band (Fig. 3, Table 3). It could be speculated that Tt mitochondrial extract might have a PKC-type kinase that phosphorylates the 60 kD protein, and it is partially inhibited by staurosporine. However, this difference also could be due to staurosporine inhibition of phosphatases.

DISCUSSION

Endogenous kinase activity studies revealed that there is one, or more than one kinas that phosphorylates three major protein bands of 60, 46, and 35 kD. The sodium vanadate differential effect on the intensity of the 35 kD band in CMS and Ta suggests that there may be two kinases working on the 60 and 35 kD proteins and sodium vanadate works through a combination of kinase activation and/or phosphatase inhibition. Dephosphorylation of protein extracts before estimation of kinase activity lowered the intensity of the 60 and 35 kD bands, an indication of reduced kinase activity. Dephosphorylation may cause a decrease in kinase activity or make those proteins less favorable substrates for kinase activity. Staurosporine showed some differential inhibition effect on the 60 kD band only in Tt extract with no effect on the phosphorylation of the 35 kD

band. This supports the idea that the 60 and 35 kD protein bands are substrates for different kinases in Tt. Also, the 60 kD protein is subject to kinase/phosphatase activity that is different from other mitochondrial proteins it is reduced by staurosporine, whereas it was not affected in CMS, Ta, or FR. Using sodium vanadate and staurosporine as phosphatase and kinase inhibitors may give evidence that these mitochondrial proteins could have differences in their proteomes reflected in the response of their kinases or phosphatases to those inhibitors. Differential inhibition of endogenous kinase activity was reported (Kann et al, 1993; Enhersperger et al, 2001; Hawker et al, 1987). The endogenous kinase activity of the core particles of hepatitis B virus was not inhibited by staurosporine, a selective inhibitor of PKC, whereas quercetine, a selective inhibitor of the protein kinase M (PKM) did not inhibit the kinase activity during the first minute but inhibited efficiently during later phases of incubation (Kann et al, 1993). Substrate protein for phosphorylation was differentially phosphorylated by various kinasses. Major vault protein (MVP) from two mammalian cell lines (CHO and PC12 cell) was highly phosphorylated by protein kinase C in vitro and by tyrosine kinase in vivo in cell-free systems which was inhibited by EDTA in CHO cells. In contrast to CHO cells, addition of recombinant casein kinase II enhances the phosphorylation of MVP in PC12 cells (Ehrnsperger et al, 2001). Sucrose phosphatase was found to be more sensitive to inhibition by sodium vanadate and less sensitive to ammonium molybdate than was the nonspecific phosphatase in vacuole extracts of sugacane and red beet (Hawker et al, 1987). More studies are needed to detect the nature of endogenous kinases and the proteomic differences among mitochondrial proteomes of wheat lines used in this study.

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