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Identification of a co-segregative protein associated with the tillering trait in rice (*Oryza sativa* L.)

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Abstract

Tillering is an important trait for grain production in rice. To understand the mechanism underlying tillering regulation in rice, a combined proteomic and genetic approach was taken to analyze potential protein markers associated with the high-tillering dwarf phenotype. Total proteins were extracted from basal internodes of rice plants and subjected to 2-dimensional gel electrophoresis. Proteomes were compared between control rice cultivars and tillering mutant lines that are known to have a reduced number of culms or contain the *d10* allele. A total of 33 polymorphic protein markers were identified between the wild-type cultivar GLA4 and the mutant line JHCA with dwarf and high tillering phenotype. A protein spot was found to behave as a qualitative trait, cosegregating with the normal-tillering phenotype in the F_2 population. This protein was present in the wild-type plants, but was undetectable in the mutant line JHCA and the high-tillering dwarf F_2 plants. Mass spectrometry analysis identified the protein as putative carboxyvinyl-carboxyphosphonate phosphorylmutase (CPPM; EC 2.7.8.23), which may catalyze the formation of a unique C-P bond from phosphoenolpyruvate and may participate in the biosynthesis of unidentified compounds with an inhibitory effect on tillering in rice. Real-time PCR analysis revealed that the mRNA level of *CPPM* was down-regulated in the high-tillering *d10* dwarf plants, suggesting that the expression of the *CPPM* gene requires the function of *D10*. We propose that CPPM may play a role that is related to the tillering regulation in rice.

Keywords: Tillering, Carboxyvinyl-carboxyphosphonate phosphorylmutase (CPPM), Rice (*Oryza sativa* L.), Proteomics.
Abbreviations: 2-DE: two-dimensional polyacrylmide gel electrophoresis, CCD: carotenoid cleavage dioxygenase, CPEP mutase: carboxyphosphonoenolpyruvate phosphonomutase, CPPM: carboxyvinyl-carboxyphosphonate phosphorylmutase, *dad*: *decreased apical dominance*, D-D-I: decrease-decrease-increase, D: dwarf, I-I-D: increase-increase-decrease, *htd*: *high-tillering dwarf*, IEF: isoelectric focusing, MALDI-TOF MS: matrix-assisted laser desorption ionization time-of-flight mass spectrometry, MS: methionine synthase, P/A: presence or absence, PEP mutase: phosphoenolpyruvate phosphomutase, PEP: phosphoenolpyruvate, PGM: phosphoglycerate mutase, PS: position shift, PTT: phosphinothricin tripeptide, qV: quantitative variation, SL: strigolactone, TFA: trifluoroacetic acid, TI: tillering inhibitor.

Introduction

Tillers in rice (*Oryza sativa* L.) are derived from vegetative shoot branching, and the growth of tillers is one of the most important agronomic traits for rice grain production (Wang and Li, 2008; 2010). In the last decade, remarkable progress has been made in understanding the molecular basis of shoot branching control in higher plants. One of the most important discoveries in this research field has been the identification of strigolactones (SLs) as a new class of plant hormones that lead to inhibition of shoot branching (Gomez-Roldan et al., 2008; Umehara et al., 2008). SLs are originally identified as a root exudate component that stimulates parasitic plant seed germination, and have subsequently recognized as a chemical signal for the establishment of arbuscular mycorrhizal symbiosis. The roles of SLs in shoot branching inhibition have been unveiled through investigations on increased-branching mutants of various plants, including the *more axillary growth* (*max*) of *Arabidopsis thaliana*, *ramosus* (*rms*) of pea (*Pisum sativum*), *decreased apical dominance* (*dad*) of petunia (*Petunia hybrida*), and *dwarf* (*d*) or *high-tillering dwarf* (*htd*) of rice (Beveridge et al., 1994, 1996; Napoli, 1996; Morris et al., 2001; Stirnberg et al., 2002; Sorefan et al., 2003; Booker et al., 2004; Ishikawa et al., 2005; Snowden et al., 2005; Zou et al., 2005, 2006; Arite et al., 2007; Simons et al., 2007; Arite et al., 2009; Liu et al., 2009; Gao et al., 2009; Lin et al., 2009). The existence of gene orthologs between *Arabidopsis* and rice suggests that monocot and dicot species of plants share a conserved pathway for the biosynthesis of the elusive shoot branching hormone (Ishikawa et al., 2005; Arite et al., 2007; Zou et al., 2006; Wang and Li 2008). It is known that SLs are derived from carotenoid compounds (Gomez-Roldan et al., 2008; Umehara et al., 2008; Matusova et al., 2008; Alder et al., 2012). However, the research on the enzymes and reactions of SL biosynthesis is still in the infantry stage. Only four enzymes involved in the synthesis of SLs have been identified so far. They are carotenoid-cleaving dioxygenase 7 (CCD7; MAX3/RMS5/HTD1) (Booker et al., 2004; Zou et al., 2006; Johnson et al., 2006), CCD8 (MAX4/RMS1/DAD1/D10) (Sorefan et al., 2003; Snowden et al., 2005; Arite et al., 2007), Cyt P450 (MAX1) (Booker et al., 2005), and an iron-containing β-carotene isomerase (D27) (Lin et al., 2009; Alder et al., 2012). More enzymes are required for the synthesis of such complex compounds. Recently, it has been shown that the petunia DAD2, an orthologs of the rice D14 (Arite et al., 2009), may serve as the

receptor of SLs and transduce the SL signal via ubiquitination-mediated signaling pathway (Hamiaux et al., 2012). However, how the SL hormone signal is relayed to shoot branching inhibition remains largely unknown. Proteomics is an essential tool of the functional genomics. It provides very useful information from gene expression to cell metabolism and genetic linkages, and has consequently become a link among genomics, genetics, and physiology (Zivy and de Vienne, 2000). Protein markers can be useful in assessing genetic variability as well as establishing genetic distances and phylogenetic relationships among lines, species, and genus. Defining the relationships of molecular genetic markers with their physiological performance is one of the urgent and tedious tasks in the genetic research field (Xie et al., 2006). A protein plays a double role in the genetic process, serving as a gene product and a functional carrier. If proteins are treated as molecular markers for genetic analysis, they have to truly reflect genetic information from generation to generation. Rice tillers are formed on non-elongated basal internodes that independently grow from the mother stem. Many critical metabolic and regulatory activities that control the plant architecture take place in the basal internode in rice (Tanaka et al., 2005; Zhang et al., 2010). To explore the molecular mechanism underlying the tillering process in rice, we applied a combined approach of proteomics with genetic analysis to the investigation on potential protein markers of the non-elongated basal internodes that might be associated with the high-tillering trait in rice. Our results showed that a number of polymorphic protein markers existed between the proteomes of normal and high-tillering rice lines. Among them, a protein that cosegregated with the normal-tillering phenotype and was absent in the high-tillering dwarf plants was identified as putative carboxyvinyl-carboxyphosphonate phosphorylmutase (CPPM), which catalyzes the formation of an unusual C-P bond from phosphoenolpyruvate (PEP), a key step in the biosynthesis of PEP-derived biologically active complex compounds.

Results

Polymorphisms in the proteome of basal nodes between JHCA and GLA4

Total proteins were extracted from basal nodes of different rice cultivars and mutant lines (Fig. S1) and used for generating 2-DE maps. There were approximately 1,100 protein spots per gel on average. Most proteins were present within a pH range of 4 to 8 and had a molecular mass range of 20 to 100 kDa (Fig. 1 and Fig. S2). The 2-DE maps of JHCA, GLA4, and their hybrid F_1 were compared using PDQuest software, and a total of 33 protein spots with differential expression were detected. These protein spots were assigned codes from B1 to B34, with spot B21 served as a reference in 2-DE maps (Fig. 1 and Fig. S2). These polymorphic proteins were divided into two groups, characterized by qualitative and quantitative variations among JHCA, GLA4, and their hybrid F_1 (Fig. 2 and Table 1). Twelve protein spots were detected only in JHCA but not in GLA4. Thirteen unique spots were found in GLA4, but not in JHCA. These 25 protein spots showed qualitative variations and could be scored as presence or absence (P/A) between the two 2-DE maps. Eight spots showed quantitative variations (qVs) in protein abundance on the 2-DE maps between JHCA and GLA4. Among these qVs, four proteins (B1, B2, B7, B22) were more abundant in JHCA than in GLA4, whereas four other proteins (B3, B5, B6, B19) were vice versa. Interestingly, all these differentially-expressed proteins were found in the F_1 plants (Fig. 1), suggesting that they were expressed in a co-dominant manner.

Common polymorphic protein markers associated with the high-tillering dwarf trait

We reasoned that potential protein markers associated with the high-tillering dwarf trait should appear as common polymorphisms in both pairs of JHCA (MT) - GLA4 (WT) and XJC (MT) - GC13 (WT). We then compared the two lists of polymorphisms, and identified 14 common polymorphic protein markers (Table 1, Fig. 3, and Fig. S3). The differential-expression patterns of these proteins in XJC and GC13 were similar to those in JHCA and GLA4.

Determination of polymorphic protein identities by MALDI-TOF/MS

The 14 protein spots associated with the high-tillering dwarf trait were subjected to proteomic identification by MALDI-TOF/MS. Five proteins corresponding to 12 protein spots were successfully identified (expect reference spot B21, Table 2). As illustrated in Fig. 2 and Table 2, B20 and B29 were allelic to B23 and B30, respectively. These four spots (B20, B23, B29 and B30) belonged to two polymorphic markers with position shift (PS) variations. Seven qV type protein spots (B1, B2, B3, B5, B6, B7, and B22) were found to correspond to three proteins. Among them, two proteins were represented by three spots each, i.e., B1-B2-B3 and B5-B6-B7. The three isospots in each family were present contiguously at the horizontal level on the 2-DE maps (Fig. 2 and Table 2), and showed a reciprocal pattern of differential expression between MT and WT. The spot family B1-B2-B3 was identified as putative vitamin B12-independent methionine synthase (EC 2.1.1.14), or 5-methyltetrahydropteroyl triglutamate-homocysteine methyltransferase. It showed a pattern of protein expression levels as increase-increase-decrease (I-I-D) in MT lines (JHCA and XJC), but decrease-decrease-increase (D-D-I) in WT plants (GLA4 and GC13). The other spot family B5-B6-B7 was identified as putative phosphoglycerate mutase (PGM; EC 5.4.2.1) and showed a pattern of differential expression as D-D-I in MT, but I-I-D in WT. In F_1, the three isospots of these two spot families showed a pattern of I-I-I. Interestingly, B20, B22 and B23 were all identified as flavone O-methyltransferase. Different allelic spot(s) of a protein or isospots of spot families were scored as one variation. Thus, a variation could mean a single P/A spot, a qV spot, a pair of two allelic PS spots, or a spot family with three isospots. A total of eight common polymorphic protein markers were found between the two MT–WT groups, including two PSs (B20-B23 and B29-B30), three P/As (B27, B28, and B31), and three qVs (B1-B2-B3, B5-B6-B7, and B22). We hypothesized that some of these polymorphic proteins may be closely related to the high-tillering dwarf trait. Therefore, the proteomes of F_2 plants with the high-tillering dwarf mutant phenotype from the cross between JHCA and GLA4 were further investigated.

Cosegregative protein in F_2 mutant plants

Eight F_2 plants with the high-tillering dwarf mutant phenotype, SM1 to SM8, were randomly selected for proteome analysis. The segregation patterns of the eight protein markers were found to be different in the eight mutant plants (data not shown). Only one, B31, cosegregated with the high-tillering dwarf mutant trait (Fig. 4A). Spot B31 was absent in the maternal parent JHCA (MT) and all eight F_2 mutant plants, but was present in the paternal parent GLA4 (WT) and the hybrid F_1. The distribution patterns of B31 on 2-DE maps in the F_2 population from the cross between XJC and GC13 were similar to those of the cross between JHCA and GLA4. As showed in Table 2, B31 was

identified as putative carboxyvinyl-carboxyphosphonate phosphorylmutase (CPPM, EC 2.7.8.23), also known as carboxyPEP mutase. This enzyme catalyzes transfer of the carboxyphosphonate group from the hydroxyl group to the C3 of phosphoenolpyruvate (PEP), leading to the formation of a unique C-P bond (see below). This reaction is a key step in the biosynthesis of the potent herbicide phosphinothricin tripeptide (PTT) in *Streptomyces hygroscopicus*. PTT is a chemical analog of glutamine and is a potent inhibitor of glutamine synthetase of plants.

Expression of B31 in rice cultivars and mutant line

In order to determine if B31 was expressed in other rice cultivars and mutant lines, we analyzed three rice cultivars, AJNT, Nippobare and TP309, and a mutant line SND with reduced culm numbers. B31 was highly expressed in all these rice cultivars and mutant line (Fig. 4B).

Transcripts of D10 and CPPM

In order to determine if the *D10* and *CPPM* genes were regulated at the transcriptional level, we measured their mRNA transcripts by real-time RT-PCR. As shown in Fig. 5, the level of mutated *d10* transcripts was drastically elevated in both *d10* mutant lines, JHCA and XJC, suggesting that *D10* expression is subjected to feedback regulation, which is consistent with observations by other researchers (Arite, 2007; Minakuchi, 2010). The transcript of *CPPM* was significantly down-regulated in JHCA and XJC as compared with that in the wild type cultivars (Fig. 5), suggesting that a positive correlation between protein content and the level of its mRNA transcript. It remains to be determined how the expression of *CPPM* is down-regulated in the *d10* mutant lines.

Discussion

Polymorphic protein markers based on 2-DE

Proteomic polymorphisms based on 2-DE are real-time expressing markers, and can be used as an important supplemental source of molecular markers in genetic research. Applications of protein markers in genetic studies have been reported in maize (Damerval and de Vienne, 1993; Damerval and Le Guilloux, 1998), maritime pine (Costa et al., 2000), wheat (Colas des Francs et al., 1985; Amiour et al., 2003) and rice (Xie et al., 2006; Wang et al., 2008). In this report, the proteome genetic approach was applied to uncover protein marker polymorphisms of basal nodes correlated closely with the high-tillering dwarf trait in rice. Three types of variations, PSs, P/As, and qVs, were scored in this work. In the 2-DE patterns, spots exhibiting qualitative variations (PSs and P/As) were shown to be under monogenic control. PSs presented an inheritance of monogenic and codominant characteristics. They corresponded to allelic differences in the primary structure of a protein given that they differed in their isoelectric points owing to mutations at the structural locus. We scored four of such spots belonging to the two structural genes, encoding flavone O-methyltransferase (B20 and B23) and putative formate dehydrogenase (B29 and B30). Three P/As were found, but only B31 was identified as CPPM by MALDI-TOF/MS. The P/As were ambiguous because they may correspond either to position shifts (PS) where a member of the pair is not detected, or to a quantitative variation (qV) as a consequence of the action of regulatory genetic elements, where the absent spot is below the level of detection by silver staining. Alternatively, the absence of a spot could correspond to a null-allele. Therefore, a P/A locus corresponds to either the structural gene of the protein or a regulatory gene controlling the expression levels of the protein (Plomion et al., 1997). Nevertheless, their genetic base can be monogenic, and they can thus be used as dominant markers (Bahrman and Damerval, 1989). The qVs can be under the control of multiple loci (Damerval, 1994). qVs have previously been shown to have a genetic basis typically, being inherited in a Mendelian manner (Bahrman and Damerval, 1989). Dominance was observed for most qVs, and in most cases, the more abundance of a protein spot is a dominant trait over the less abundance of the protein. This finding was consistent with previous observations by other groups, which show that the relative abundances of proteins in the F_1 hybrids, on average, deviate towards the high parental values (Leonardi et al., 1988; de Vienne et al., 1988). In addition, we also observed that the expression patterns of the three isospots in the two spot families, B1-B2-B3 and B5-B6-B7 showed a reciprocal relationship between MT and WT. The B1-B2-B3 spot family was identified as putative methionine synthase (MS), and B5-B6-B7 as putative phosphoglycerate mutase (PGM). This result needs to be further investigated in order to determine whether this reciprocal regulation is related to the tillering control in rice. Except for B31, other polymorphic proteins did not have an exact cosegregative relationship with the high-tillering dwarf phenotype in mutant plants.

Cosegregation of CPPM with the high-tillering dwarf trait

The spot image that a polymorphic protein may create in a 2-DE pattern is considered to be a phenotype of that protein (Klose et al., 2002). By comprehensive analysis, CPPM (B31), a P/A variation protein marker, was found to cosegregate with the high-tillering dwarf trait of mutant plants. It showed "absence" in all mutant plants, but "presence" in all WT cultivars as well as in the reduced culm number mutant SND. These results suggested that CPPM might participate in a pathway that produces a compound that has inhibitory effects on tillering in rice. CPPM is also known as carboxyphosphonoenolpyruvate phosphonomutase (CPEP mutase) (Brock et al., 2001), and belongs to the isocitratelyase/phosphoenolpyruvate phosphomutase (PEP mutase) enzyme superfamily (Joosten et al., 2008). The genes and biochemical characteristics of CPPM have been investigated mainly in the gram-positive bacterium *Streptomyces hygroscopicus* (Hidaka et al., 1990, 1992). CPPM is evolutionarily related to PEP mutase (EC 5.4.2.9), which catalyzes the transfer of a phosphoryl group ($-H_2PO_3$) within the PEP molecule. In comparison, CPPM catalyzes the transfer of a carboxyphosphonate group ($-HPO_2COOH$) within the PEP molecule (Fig. 6). The reaction by CPPM leads to the formation of a unique C-P bond, which is a key step in the biosynthesis of the potent herbicide PTT in *Streptomyces hygroscopicus* (Schomburg et al., 2008; Hidaka et al., 1990). Both CPPM and PEP mutase are known to be required for the biosynthesis of PTT (Hidaka et al., 1990; Metcalf and van der Donk, 2009). It is possible that a PTT-like phosphonate compound may serve as a tillering inhibitor (TI) in rice (Fig. 6). There have been very limited investigations on the biosynthesis of phosphonates and their existence in higher plants. In the oligomeric stromal proteome of *Arabidopsis thaliana* chloroplasts, two 33-kDa paralogs, At1g21440 and At1g77060, encoding putative plastid-localized CPEP mutases have been indentified (Peltier et al., 2006). However, the biochemical reaction and biological functions of these CPEP mutases in *Arabidopsis* have not been studied. Wang et al., (1993) have isolated a cDNA clone (pSR132) representing an mRNA that accumulates in senescing carnation flower petals in response to ethylene. A comparison of the predicted peptide sequence of pSR132 reveals a significant homology with CPEP and PEP mutases in *Streptomyces* and

Table 1. Polymorphic protein spots between JHCA and GLA4.

Spot variation	Protein abundance	Differentially expressed proteins
Qualitative	Only in JHCA	B4, B9, B11, B12, B13, B17, B20[a], B24, B25, B27[a], B29[a], B33
	Only in GLA4	B8, B10, B14, B15, B16, B18, B23[a], B26, B28[a], B30[a], B31[a], B32, B34
Quantitative	More in JHCA	B1[a], B2[a], B7[a], B22[a]
	More in GLA4	B3[a], B5[a], B6[a], B19

[a] Common polymorphic protein markers between the high-tillering dwarf line JHCA and the normal-tillering cultivar GLA4, and between the high-tillering dwarf line XJC and the tall-culm cultivar GC13.

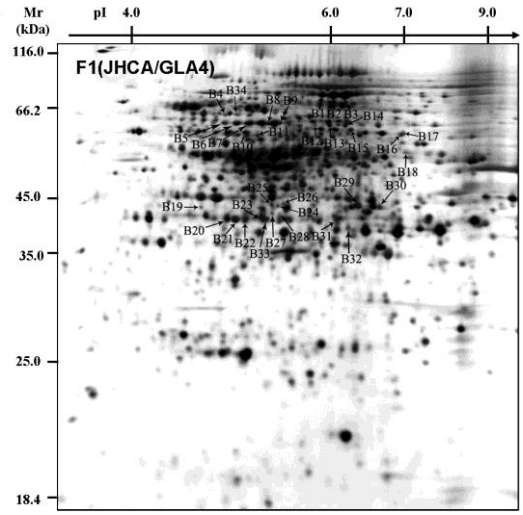

Fig 1. Proteomic profile of basal nodes in hybrid F_1 at the active tillering stage. Total proteins were isolated from the basal nodes of F_1 plants, and subjected to 2-D gel electrophoresis. Spots of differentially expressed proteins between two parents, JHCA and GLA4, are indicated by arrows and assigned serial codes.

Tetrahymena, respectively. Given the nature of the reactions catalyzed by CPEP and PEP mutases, pSR132 could play a catabolic role by degrading phosphonolipids released from the membrane during senescence. In SL pathway-related mutants (*max/dad/ore/d/htd*), previous studies have shown that the *dad1/max4/d10* mutant in *Petunia* (Snowden et al., 2005) and *d3/max2/ore9* mutant in rice (Yan et al., 2007) have delayed leaf senescence phenotypes. Therefore, it is also possible that CPPM may be involved in the organ senescence or cell death in plants by affecting cell membrane stability. The *D10* gene in rice encodes the carotenoid cleavage dioxygenase 8 (CCD8) enzyme, which catalyzes a key step in the biosynthesis of SLs from carotenoid compounds (Arite et al., 2007; Umehara et al., 2008). Both high-tillering dwarf lines JHCA and XJC are *d10* mutants and their phenotypes can be rescued by supplementation with GR24, a synthetic SL analog (data not shown). Previous work by others (Arite et al., 2007) and our real-time PCR analysis have showed that the levels of *d10* mRNA are drastically up-regulated in both JHCA and XJC lines, suggesting that the level of *D10* mRNA accumulation might be a critical step in the regulation of the synthesis of SLs, the branching inhibitor. This work was focused on the differentially-expressed proteins that could be detected in 2-DE. Our proteome analysis of JHCA and XJC lines did not detect differential expression of D10 at the protein level, possibly due to the low abundance of D10 or the resolution limit of 2-DE. We propose that CPPM may act as a downstream target of SLs, the tillering inhibitor in rice (Fig. 6). SLs are synthesized in roots and translocated via xylem to the shoot where they exert the inhibitory effects on branching (Gomez-Roldan et al., 2008; Umehara et al., 2008; Domagalska and Leyser, 2011). SLs represent a new class of plant hormones and are likely to regulate shoot branching through specific signal transduction pathways as other classic plant growth regulators do. CPPM could be one of the target enzymes that are regulated by the SL-signaling pathway (Fig. 6). Our real-time PCR analysis showed that the *CPPM* gene expression is down-regulated in both JHCA and XJC mutant lines, suggesting that the expression of *CPPM* requires the function of D10 and the SL-signaling pathway. In the absence of a functional *D10* gene in JHCA, XJC and their F_2 mutant plants, no SLs are produced, leading to the lack of *CPPM* gene expression and "absence" of CPPM protein on 2-DE gels. It is not known if the abundance of CPPM protein is also regulated by D10 and SLs at the post-transcriptional level. It would be interesting to further characterize the rice CPPM at the biochemistry level. It would also be very important to investigate how CPPM exert its role in tillering control in rice. This work has demonstrated the potential of combining proteomics with genetic analysis in rice breeding program.

Materials and methods

Plant material and sampling

Five wild-type (WT) cultivars and three mutant lines obtained from the rice collection at the Institute of Genetics and Rice Breeding, Xiamen University, were used in this work. The WT cultivars including the semi-dwarf indica rice GLA4 and AJNT, the tall-culm indica rice GC13, the semi-dwarf japonica rice Nippobare, and the tall-culm japonica rice TP309, exhibited normal tillering traits. The three mutant lines used in this work included the japonica mutant 'SND', which had a reduced number of culms, and two allelic *d10* mutants, JHCA and XJC, which showed a high-tillering dwarf phenotype. The mutant line JHCA (Fig. S1) was originally isolated from an M_2 mutation population generated by gamma (γ) −ray irradiation. Our earlier

Table 2. Identification of polymorphic proteins by peptide mass fingerprinting.

GenBank Acc. No.	Protein name	Mr (Da) / p*I*[a]	Protein Spot[b]	MP[c]	SC[d] (%)	Scores
Q2QLY5	Putative vitamin B12-independent methionine synthase	85/5.9	B1	14	27	56
			B2	15	30	66
			B3	20	35	106
Q5QMK7	Putative phosphoglycerate mutase	61/5.4	B5	19	38	120
			B6	19	36	160
			B7	22	50	178
Q19BJ6	Flavone O-methyltransferase	40/5.4	B20	21	57	128
			B22	13	33	77
			B23	13	42	68
AJRZQG	Glutamate-ammonia ligase	39/5.5	B21[e]	16	53	127
EEC80636	Putative formate dehydrogenase	41/6.6	B29	16	45	101
			B30	17	41	104
Q2QWN6	Putative carboxyvinyl-carboxyphosphonate phosphorylmutase	38/6.1	B31	13	32	63

[a] Mr, theoretical relative molecular mass in kD; p*I*, theoretical isoelectric point. [b] Differentially expressed protein spots were excised from 2-DE gels and subjected to peptide mass fingerprinting with MALDI-TOF/MS. [c] MP, matched peptides. [d] SC, sequence coverage. [e] Reference protein

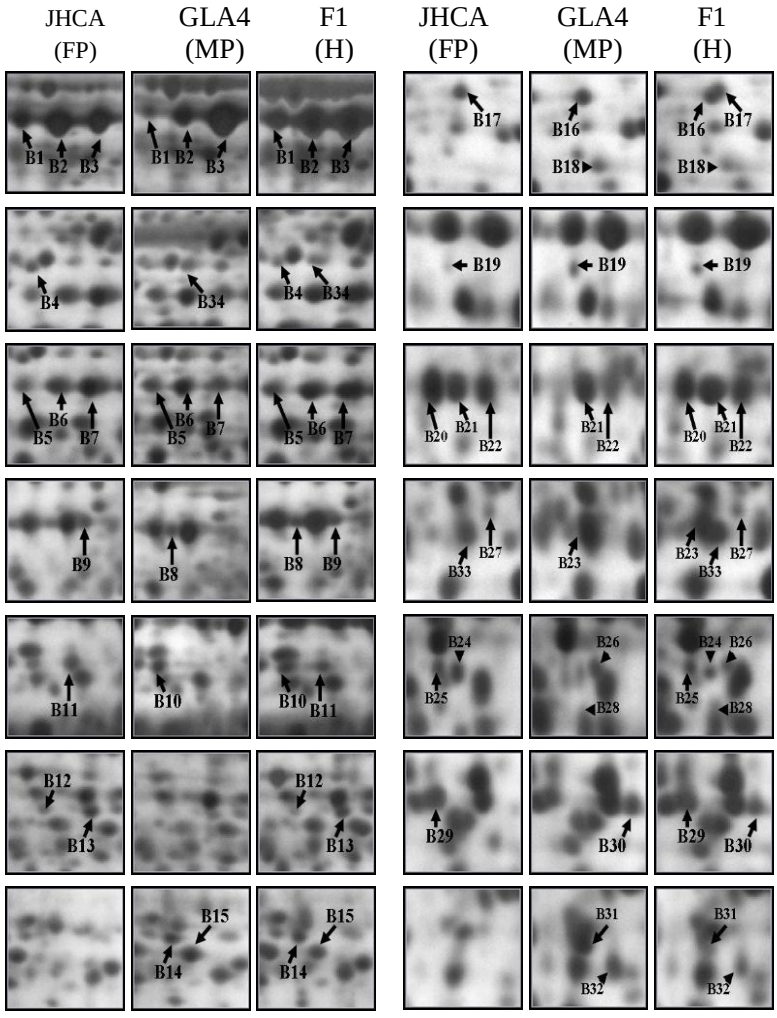

Fig 2. Differential expression of proteins among JHCA, GLA4 and hybrid F_1. FP, MP and H denote female parent, male parent and hybrid, respectively. Arrows indicate the positions of protein spots showing differential expression.

Table 3. Segregation analysis of F_2 populations derived from crosses between JHCA and GLA4.

Cross (Female/male)	No. of plants			χ^2_C (3 : 1) ($\chi^2_{0.05}$ = 3.84)
	WT	Mutant	Total	
JHCA/GLA4	325	114	439	0.171
GLA4/JHCA	278	89	367	0.074

For genetic analysis, JHCA was crossed with GLA4, a semi-dwarf cultivar. Plants of the F_1 generation exhibited a normal tillering, semi-dwarf phenotype. Plants of the F_2 populations were scored as wild-type (WT, normal tillering, semi-dwarf) and mutant (high-tillering, dwarf) on the basis of phenotypes.

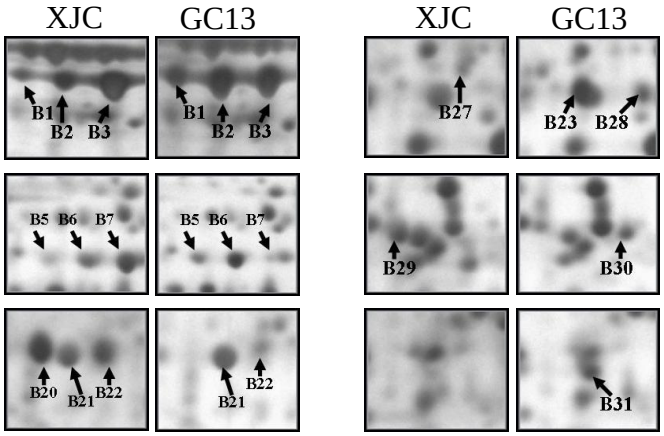

Fig 3. Differential expression of proteins between XJC and GC13. Total proteins were extracted from the high-tillering dwarf line XJC and the tall-culm cultivar GC13 and resolved by two-dimensional polyacrylamide gel electrophoresis maps. The corresponding portions of the 2-DE gels were cropped for comparison. Differentially expressed polymorphic proteins between XJC and GC13 are indicated.

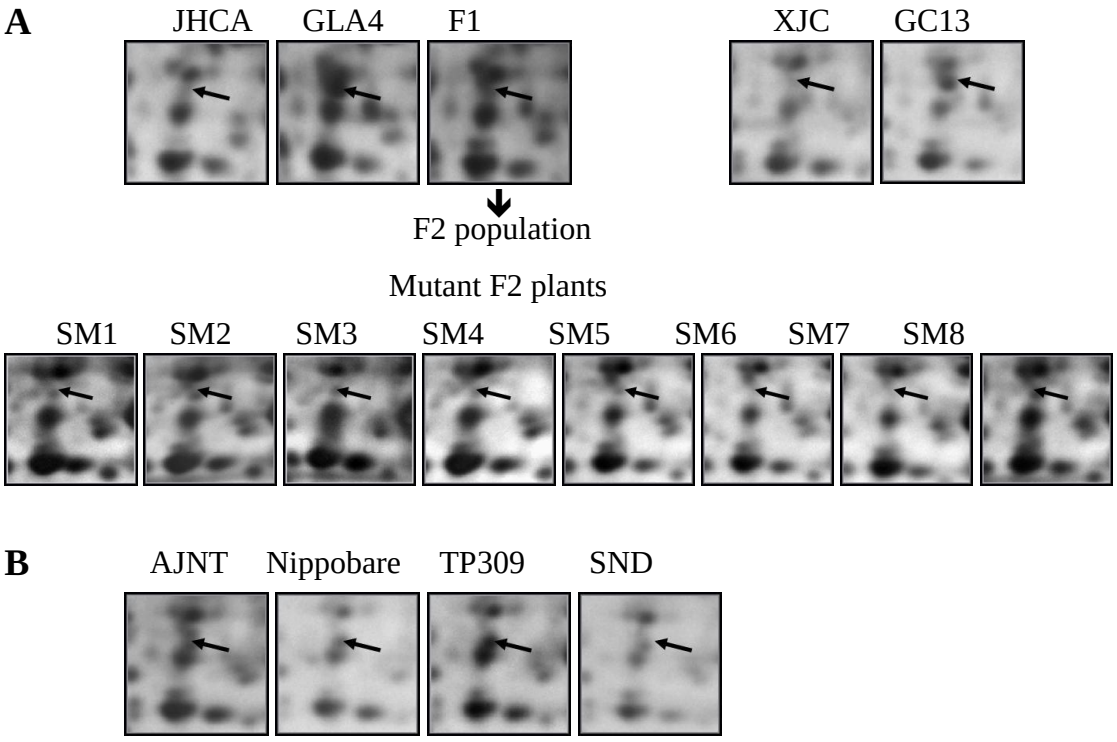

Fig 4. Genetic pattern of protein spot B31. (A) Spot B31 was detected in the normal-tillering cultivar GLA4, the tall-culm cultivar GC13, and F_1 plants of the cross between JHCA and GLA4. This protein was absent (undetectable) in the high-tillering dwarf lines JHCA and XJC, and in the F_2 plants with the high-tillering dwarf phenotype (SM1 to SM8) from the cross between JHCA and GLA4. (B) Spot B31 was detected in three other wild-type cultivars (AJNT, Nippobare and TP309) and the reduced culm number mutant SND.

studies have demonstrated that JHCA carries two dwarf-related genes, *sd1* and *d10* (our unpublished data). The recessive *d10* locus contains a 39-bp deletion at the second exon of *D10* (LOC_Os01g0746400) resulting in frame-shift mutation (data not shown). The allelic mutant line, XJC, was isolated from the offspring of the cross between JHCA and GC13, and possessed only the recessive *d10* gene. After inbreeding for several generations, XJC attained genetic stability and exhibited semi-dwarf plant height and a high-tillering phenotype (Fig. S1). More than 120 tillers could be observed in JHCA and XJC at the maturing stage (Fig. S1). The F_2 population characterized in this work was generated from the cross between JHCA (female parent) and GLA4 (male parent). The tillering traits and plant height of the F_1 generation were similar to that of GLA4 (Fig. S1). Eight F_2 plants with the high-tillering dwarf phenotype were randomly selected for proteomic analysis. Pre-germinated rice seeds were sown in a field in March 2010, and the seedlings were transplanted to a density of approximately 20 cm × 20 cm per plant.

Preparation of total proteins from rice basal nodes

For proteomic analysis, 0.5-cm sections from the basal nodes were harvested at the active tillering stage, or about 30 days after transplanting, according to Yeu et al., (2007) with some minor modifications. The rice basal nodes collected from different cultivars were ground to fine powders with a mortar and pestle pre-immersed in liquid nitrogen. Total proteins were extracted in extraction buffer and precipitated by trichloroacetic acid as described by Damerval et al., (1986). The pellets were dissolved in a concentration of approximately 40 µL/mg protein in the O'Farrells UKS buffer (9.5 M urea, 5 mM K_2CO_3, 1.25% sodium dodecyl sulfate (SDS), 0.5% DTT, pH 3.5 to 10.0 2% pharmalyte, and 6% Triton X-100) (O'Farrell, 1975). To facilitate protein desolation, the pellet-buffer mixture was shacked at room temperature for 1 h, followed by centrifugation at 15,000×g for 30 min at 15 ^{o}C. Protein content of the soluble fraction was determined using the BCA Protein Assay (Pierce). The soluble fraction was used for two-dimensional polyacrylmide gel electrophoresis (2-DE) or stored at -80 ^{o}C.

Two-dimensional electrophoresis

Isoelectric focusing (IEF) was carried out in 15 cm tube gels (inner diameter 2 mm) containing 8 M urea, 3.5% acrylamide, 2% Nonidet P-40, and 2% ampholine (pH ranges 3.5-10 and 5-8, 1:4, v/v). Approximately 150 µg of proteins per sample were applied to one gel. IEF was run for 15 min at 200 V, followed by 30 min at 300 V, 30 min at 400 V, 16 h at 600 V, 30 min at 800V, and 30 min at 1000 V. The gels were equilibrated in an equilibration buffer (pH 6.8 125 mM Tris buffer, 40% w/v glycerol, 65 mM dithiothreitol (DTT), and 3% w/v SDS) twice for 15 min each. The gels were then subjected to the second dimension SDS-polyacrylamide gel electrophoresis (PAGE), which was performed on 12% slab gels (160 mm long and 1 mm thick) at 25 mA for about 5 h. The separated proteins were visualized by silver staining.

Silver staining

SDS-PAGE gels were incubated for 30 min in an sensitization solution (68 g/L sodium acetate, 2.0 g/L sodium thiosulfate, 30% v/v ethanol), washed three times for 5 min each in distilled water, and incubated in 0.25% $AgNO_3$, 0.04% formaldehyde for a minimum of 30 min. The gels were then washed twice with distilled water for 1 min each. Protein color was developed in a 2.5% w/v Na_2CO_3, 0.02% v/v formaldehyde solution, within a

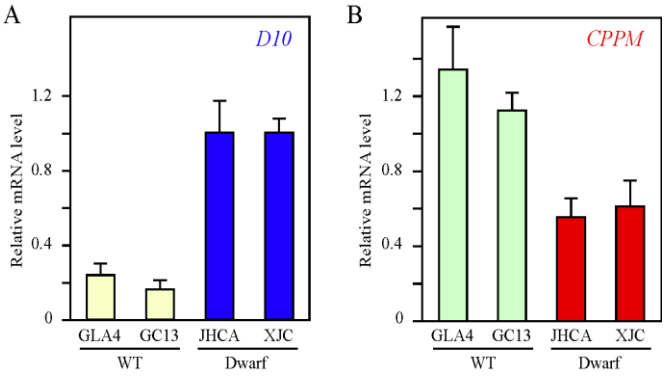

Fig 5. Real-time PCR analysis of the transcript levels of *D10* and *CPPM*. Total mRNA was isolated from the high-tillering dwarf lines JHCA and XJC, and from wild type cultivars including the normal-tillering GLA4 and the tall-culm GC13. Real-time PCR analysis was performed using gene specific primers corresponding *D10* and *CPPM*. The *OsActin* gene was used as an internal control for normalization of the data. Error bars were calculated based on three biological replicates.

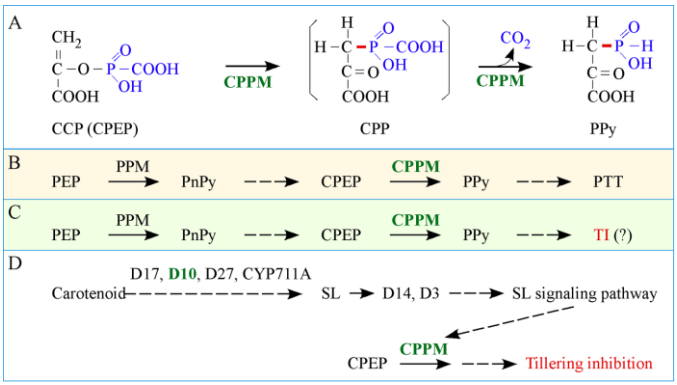

Fig 6. Proposed reaction of CPPM and its role in tillering inhibition in rice. (A) Carboxyvinyl-carboxyphosphonate phosphorylmutase (CPPM; EC 2.7.8.23), also known as carboxyPEP mutase or CPEP mutase, catalyzes the transfer of the carboxyphosphonate group (in blue) from the hydroxyl group to the C3 of phosphoenolpyruvate (PEP), forming a unique C-P bond (highlighted in red). The intermediate, carboxyphosphono pyruvate (CPP) is unstable and is subsequently converted to phosphonopyruvate (PPy), releasing CO_2. The substrate of the CPPM reaction, 1-carboxyvinyl carboxyphosphonate (CCP), is also known as carboxyPEP (CPEP). (B) In *Streptomyces hygroscopicus*, the potent herbicide phosphinothricin tripeptide (PTT) is synthesized from PEP and other metabolites through a multi-step pathway (Metcalf and van der Donk 2009). Both PEP mutase (PPM) and carboxyPEP mutase (CPPM) are required for PTT biosynthesis. PnPy, phosphonopyruvate. (C) In rice, a similar pathway could be modified to produce an unidentified tillering inhibitor compound (TI). (D) In rice, the branching inhibition hormone strigolactones (SLs) are produced from carotenoids *via* the functions of D17, D10, D27, CYP711A and other unidentified enzymes. SLs are synthesized in the roots and translocated to the shoot. SLs are perceived by D14 receptor that interacts with D3 F-box protein and initiates a SL-signaling pathway. SLs may regulate the expression of the *CPPM* gene, which may be required for the synthesis of an unidentified compound that inhibits tillering in rice.

time period of 2-5 min. Stain development was stopped with a solution that contained 14.6 g/L EDTA-Na_2. Throughout the staining procedure, gels were treated separate in 250 mL of each solution. At least three replicates were performed for each sample.

Image acquisition and data analysis

The 2-DE images were acquired under a Molecular Imager ChemiDoc XRS System (Bio-Rad) in transmission mode. Image analysis was carried out using a combination of manual visualization and software calculations of the PDQuest 7.1 2-D Analysis Software (Bio-Rad). All 2-DE images were globally analyzed by this software and the identified spots were manually checked. Significantly different spots were drawn from comparisons among the different gels.

Mass spectrometry analysis and database search

Selected protein spots detected on 2-DE gels were sliced from the gel and digested with sequencing-grade trypsin (Promega). The peptides were dissolved in 2 μL of 0.5% trifluoroacetic acid (TFA) solution and mixed with equimultiple volumes of a saturated matrix solution consisting of α-cyano-4-hydroxy-cinnamic acid in 70% acetonitrile with 0.1% TFA. The resulting mixture was then loaded onto the point template. After drying at room temperature, the template was introduced into a Bruker REFLEXTM matrix-assisted laser-desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) system (Burker, Germany) with a reflection model. The peak lists of peptide mass fingerprints were created using the FlexAnalysis 2.0 software (Bruker Daltonics), and interpreted with MASCOT (Matrix Science, London, UK., http://www.matrixscience.com/) against the MSDB and NCBInr databases.

Real-time RT-PCR

Total RNA from basal nodes was extracted using a TRIzol kit (Invitrogen). Two microgram of total RNAs was treated with DNase I and used for cDNA synthesis with Superscript III RT kit (Invitrogen). Real-time PCR were performed using the following gene-specific primer sets: forward and reverse primers for *D10*, 5'-CGTGGC GATATC GATGGT-3' and 5'-CGACCT CCTCGA ACGTCTT-3'; for *CPPM*, 5'-ATCGGG ACTTCT TTATCG TGG-3' and 5'-TTGCAC ACGCGA TAACCC-3'; and for *OsActin*, 5'-CTTCAT AGGAAT GGAAGC TGCGGG TA-3' and 5'- CGACCA CCTTGA TCTTCA TGCTGC TA-3'. *OsActin* was used as an internal control. PCRs were performed with SsoFast™ EvaGreen® supermix (BioRAD) using a CFX96 Real-time system (BioRAD).

Conclusion

Regulation of tillering in rice is very important for grain production. A dwarf rice mutant line with high tillering capability was investigated for potential protein markers associated with the tillering phenotype. A novel protein with homology to CPPM was identified as a cosegregative protein associated with the normal-tillering trait in rice. The CPPM protein was undetectable in the high-tillering dwarf lines and in the F_2 plants with the mutant phenotype. CPPM has no known function in the primary metabolism in bacteria and plants. It may play a role in the secondary metabolism. On the basis of homology to the *Streptomyces* CPPM, it is proposed that the rice protein may be required for the formation of unusual C-P bonds that are present in unidentified compounds with a tillering inhibitory activity in plants. This work also demonstrates the potential of applying proteomic approaches to genetic analyses and breeding practice in rice research.

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Supplementary materials are available online.

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