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Map-based Cloning of Panicle Apical Abortion and Dwarfing Mutant Genes in Rice

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Map-based cloning of panicle apical abortion and dwarfing mutant genes in rice

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GENERAL ABSTRACT

Rice (*Oryza sativa* L.) is one of the most important food crops in the world and also staple food for heavily populated Asian countries as well as many African countries. Crop production and productivity must be increased to provide a balanced diet for the global population. Plant architecture is considered as a viable approach, because crop plants with desirable architecture are able to increase grain yield. Panicle structure and plant height are major components of plant architecture and determinate of grain production in rice.

The panicle architecture is primarily determined by the arrangement of branches and spikelets, and it directly affects grain yield. We identified a mutant for panicle apical abortion (PAA-Hwa) from a *japonica* cultivar Hwacheongbyeo treated with N-methyl-N-nitrosourea. Under normal growth conditions, the mutant had multiple abnormal phenotypes, such as a slight reduction in plant height, narrow and dark green leaf blades, and small erect panicles with clear panicle apical abortion compared to the wild-type plants.

Genetic analysis revealed that the panicle apical abortion was controlled by a single recessive gene, which is tentatively designated as *paa-h*. The *paa-h* gene was fine mapped at an interval of 71 kb flanked by STS markers aptn3 and S6685-1 at the long arm of chromosome 4. Sequence analysis of the candidate genes within the delimited region, *LOC_Os04g56160* gene showed a single base-pair change corresponding to an amino acid substitution from glycine to glutamic acid. The *LOC_Os04g56160* gene encodes a Plasma membrane ATPase protein containing 951 amino acids. The T-DNA mutant lines of *LOC_Os04g56160* gene exhibited the same phenotype of the original PAA-Hwa mutant, which confirmed the function of *LOC_Os04g56160* gene for the panicle apical abortion phenotype in rice.

Dwarfism is one of the most important agronomic traits in crop breeding because dwarf cultivars are more resistant to lodging and led to the remarkable yield increases in monocot plants, especially in rice. However, the genetic mechanisms controlling dwarfism are not well characterized, and causal genes underlying most dwarf mutants are remain uncovered. We characterized a dwarf13 rice mutant derived from Shiokari by multiple rounds of back-crossings. Phenotypic analysis under the field growth condition indicated that the mutant had shown several altered phenotypes comparing to wild type plants, such as reduced plant height, increased tiller number, short and rounded leaf tip, yellowish stripe on leaf blades, late and asynchronous heading, strong root systems, short erect panicle, smaller seed size, etc. The mutant was categorized as dn-type dwarf mutant according to the elongation pattern of internodes by Takeda. In addition, the dwarfing of the dwarf13 mutant was responsive to GA, based on the analyses of two GA-mediated processes belongs to group E by Mitsunaga. The expression levels of gibberellin (GA) biosynthetic genes including OsCPS1, OsKS1, OsKO1,

OsKAO, OsGA20ox2, OsGA2ox3 and OsGA2ox1 were significantly increased in dwarf13 mutant. Anatomical observations revealed that the dwarf13 mutant had less number of vascular bundles and higher number of smaller size cell than wild type plants, elucidation the dwarf phenotype. Genetic analysis indicated that the dwarf13 mutant was controlled by a single recessive gene. By fine-mapping strategy, *d13* gene was flanked by two STS markers namely, DMR-3 and S5789 within the physical distance of 265 kb in the centromeric region of chromosome 9. Based on available sequence annotation databases, there are thirty predicted genes annotated in this region.

We expect that the *paa-h* and dwarfing mutant gene will be a new clue for the molecular mechanism of panicle apical abortion and dwarfism to maintain the grain yield production in rice breeding programs.

Keywords: Rice, Panicle apical abortion, Plasma membrane ATPase, Dwarfism, Gibberellin (GA), Map-based cloning

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LIST OF ABBREVIATIONS

PAA	Panicle apical abortion
BAC	Bacterial artificial chromosome
BSA	Bulked segregant analysis
dCAPS	Derived cleaved amplified polymorph sequence
STS	Sequence tagged site
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism
TBE	Tris-borate-EDTA
DBH	Days before heading
HD	Heading date
CL	Culm length
PN	Panicle number per hill
SN	Spikelet number per plant
WT	Weight
SF	Spikelet fertility
GL	Grain length
GW	Grain width
GS	Grain shape
GA	Gibberellin
MNU	N-methyl-N-nitrosourea
SSR	Simple Sequence Repeat

GENERAL INTRODUCTION

Rice (*Oryza sativa* L.) is one of the world's oldest and most important crop species, having been domesticated beginning approximately 8,000–9,000 years ago (Higham and Lu, 1998; Liu et al. 2007). Its long history of cultivation and selection under diverse environments, acquired a broad range of adaptability and tolerance so that it can be grown in a wide range of water/soil regimens from deeply flooded land to dry hilly slopes (Lu and Chang 1980). Through a process of continuous selection for desirable features, the early farmers slowly transformed wild rice into *Oryza sativa*.

Rice is one of the most important food crops in the world and also staple food for heavily populated Asian countries as well as many African countries. The Food and Agricultural Organization (FAO) estimate that 925 million people were undernourished worldwide during 2010 compared with 1.023 billion during 2009, while the ongoing financial and economic crisis could force even more people into hunger and poverty (FAO 2010). The food grain production rate should be increased to overcome poverty and hunger, especially in cereals and legumes, because about 50% of the calories consumed by the world population are in the form of cereals such as rice (23%), wheat (17%) and maize (10%) (Khush 2003).

The global population is increasing every day, so the rate of food crop productivity will have to be increased to feed the world population. Improving crop productivity by selection for the components of grain yield and optimal plant architecture has been the main focus in rice breeding (Wang and Li 2008; Zhang 2007). In rice breeding programs, Panicle structure and plant height are two major components of plant architecture and determinate of grain production.

Rice panicle architecture not only contributes tograin yield, but also to the ecological conditions of cultivated populations and the physicochemical properties of different varieties (Xu et al. 1996; Yuan 1997; Chen et al. 2001). Diverse inflorescence architectures are common in flowering plants. In the rice inflorescence development, three major factors, namely, timing of IM abortion, conversion of the rachis branch meristem to the terminal spikelet meristem and programme shift of lateral meristem identity along a basal-apical direction on a rachis branch, determine the overall architecture of the rice panicle (Ikeda-Kawakatsu et al. 2009). The rice panicle consists of a number of primary and secondary rachis branches, which initiate spikelets (Yamagishi et al. 2003; Bommert et al. 2005; Mu et al. 2005). The potential number of spikelets in rice is determined during these events, but the abortion of young spikelets before heading reduces spikelet number per panicle (Sheehy et al. 2001; Ansari et al. 2003; Yamagishi et al. 2004). This is called pre-flowering abortion (Senanayake et al. 1991), which frequently occurs at the basal part of each primary rachis branch (Matsushima 1966) contributing yield loss. However, there have been very few reports about panicle apical abortion.

Plant height is a very important agronomic trait in crop breeding conferring resistance to lodging and contributing to stable high yields (Evans 1998; Conway and Toenniessen 1999; Khush 2001). The most striking example arose in the late 1950s, when selection for the semi-dwarf stature in rice and wheat greatly improved plant architecture and yield potential (Peng et al. 1999; Monna et al. 2002; Sasaki et al. 2002; Spieimeyer et al. 2002). Besides dwarf mutant, the utilization of dwarf and semi-dwarf genes, together with the wide spread use of fertilizers and pesticides, led to the remarkable yield increases in monocot plants, especially in rice

(Hedden2003). It is mainly a result of deficiency in certain endogenous plant hormones, such as gibberellins (GAs) and brassinosteroids (BRs) (Fujioka and Yokota 2003; Yamaguchi 2008), or modification of their signaling transduction (Sun et al. 1992; Peng et al. 1997).

Rice grain shape also plays a major role in consumer preferences in different countries and populations. In the USA, Southern China and most Asian countries, people prefer long and slender grain varieties, whereas people from South Korea, Japan, Northern China and Sri Lanka prefer short, rounded grain varieties (Weng et al. 2008; Bai et al. 2010; Shao et al. 2010).

Dwarf mutants have been isolated in many species and also extensively studied in *indica* and *japonica r*ice to understand the essence of dwarfing mechanism; although some are the same genes or allelic (Ashikari et al. 1999; Mori et al. 2002; Hong et al. 2003), but among them very few are small grain dwarf and only the *sd-1* gene has been used widely in rice breeding. So the extensive use of limited dwarfing sources may cause a 'bottleneck' effect in genetic background for breeding new rice varieties (Hargrove and Cabanilla 1979). However, only a few small grain dwarfing genes have been identified and the dwarfing mechanisms are still remaining unclear. We expect that our studied mutant gene will be a new clue to uncover the dwarfing mechanism and also helpful to develop a small grain high yielding variety in rice breeding.

The objectives of these studies are identification, characterization and elucidate the molecular mechanisms of the panicle apical abortion and dwarfism in rice mutant through map-based cloning.

LITERATURE REVIEW

Map-based cloning of mutant gene

Mutants are valuable resources for genetic variations in crop improvement. The novel genetic variations obtained from either spontaneous or induced mutants using physical or chemical mutagens can be exploited in crop genetics and their application in functional genomics and molecular breeding (Krishnan et al. 2009; Jiang and Ramachandran 2010). Analysis of genetic mutations is one of the most effective techniques for investigating gene function. Genes controlling developmental and metabolic processes have been discovered in plants by mutational analysis (Miroslaw and Iwona 2003).

Generally established techniques for gene isolation presuppose knowledge of the transcript or the protein product of the gene. However, for most genes this information is not available. Knowledge of the gene location on a genetic linkage map offers an alternative method of gene cloning. Map-based cloning consists of three general steps, firstly, identification of markers that show tight genetic linkage to and that flank the target gene. Second step, a walk to the gene is undertaken by using various genomic libraries constructed in, like yeast artificial chromosome vectors, finally confirmation requires the comparison of the isolated gene with a wild type or, in the case of plants, complementation of the recessive phenotype by transformation. Successful cloning of human disease genes has validated the map-based cloning approach (Martin et al. 1991).

Inflorescence development and different architecture in plants

Inflorescence architecture is a key agronomical factor determining grain yield, and thus has been a major target of crop improvement (Gallavotti et al. 2004; Huang et al. 2009). Understanding the genetic basis of inflorescence architecture of mutant will contribute to improve crop grain yield. To unravel mechanisms regulating panicle architecture approaches such as genetic and functional analysis of inflorescence mutants would enable the exploitation of inflorescence characteristics for yield improvement (Rao et al. 2008; Kellogg 2007).

Plants undergo a series of complicated events to form the specific panicle structure during development. All inflorescence structures are generated from inflorescence meristems, which are defined by the WUS-CLV feedback regulatory loop in arabidopsis (Schoof et al. 2000). Almost three decades of genetic and molecular analyses have resulted in detailed insights into many of the processes that take place during flower development and in the identification of a large number of key regulatory genes that control these processes. Grass inflorescences comprise characteristic structural units called spikelets that can contain from one to forty florets and can be either determinate or indeterminate depending on the species (Schmidt and Ambrose 1998; McSteen et al. 2000; Goto et al. 2001). Inflorescence architecture comprises the stereotypical number and arrangement of floral branches that characterizes each species of flowering plants (Weberling 1989; Sussex and Kerk 2001). The timing of meristem phase change is crucial in the control of inflorescence architecture in grass species (McSteen et al. 2000; Bortiri and Hake 2007; Wang and Li 2008).

Eleven developmental features not discernable in the mature inflorescence were found: direction of branch differentiation; origins of primary branches; apical vs. intercalary development of the main axis; direction of spikelet differentiation; direction of glume, lemma and palea differentiation; position of the lower glume (in some cases); size of the floret meristem; pattern of distal floret development; pattern of gynoecium abortion; differential pollen development between proximal and distal floret; and glume elongation. The following developmental events are related to sex expression: size of floret meristem, gynoecium abortion, pollen development delay in the proximal floret, glume elongation and basipetal floret maturation at anthesis (Reinheimer et al. 2005). The abnormal inflorescence meristem1 (*aim1*) mutation affects inflorescence and floral development in Arabidopsis. After the transition to reproductive growth, the aim1 inflorescence meristem becomes disorganized, producing abnormal floral meristems and resulting in plants with severely reduced fertility through oxidation pathway (Richmond and Bleecker 1999).

In maize, Kernels are most susceptible to abortion during the first 2 weeks following pollination, particularly kernels near the tip of the ear. Tip kernels are generally last to be fertilized, less vigorous than the rest, and are most susceptible to abortion (Nielsen 2009). The ovules at the tip of the ear are the last to be pollinated, and under certain conditions only a limited amount of pollen may be available to germinate late emerging silks. Pollen shed may be complete before the silks associated with the tip ovules emerge. As a result, no kernels form at the ear tip. Severe drought stress may result in slow growth of the silks that prevents them from emerging in time to receive pollen. Incomplete ear fill may also be related to kernel abortion. If plant nutrients (sugars and proteins) are limited during the early stages of kernel

development, then kernels at the tip of the ear may abort. Kernels at the tip of the ear are the last to be pollinated and cannot compete as effectively for nutrients as kernels formed earlier. Stress conditions, such as heat and moisture stress, nitrogen deficiency, hail, and foliar disease damage, may cause a shortage of nutrients that lead to kernel abortion. Periods of cloudy weather following pollination, or the mutual shading from very high plant populations can also contribute to kernel abortion (Thomison 2010). Kernel abortion in maize may occur by the physiological events. Ethylene formed in the ear at pollination initiates abortion by inhibiting growth of slower growing kernels at the ear tip. Reducing sugars accumulate in the cob of aborting kernels either as a result of inhibition of kernel growth and/or inhibition of sugar transfer from cob to kernels. ABA increases in aborting kernels and may promote abortion by inhibiting both kernel growth and sugar uptake by the kernels. Approximately 1 to 4 d after initiation of kernel abortion, sugar supply to aborting kernels is terminated, kernel growth ceases and abortion is complete (Reed and Singletary 1989). Numerous mutants have been reported in maize that shows abnormalities in inflorescence and spikelets (Veit et al. 1993; Chuck et al. 1998; Chuck et al. 2002; Kaplinsky and Freeling 2003). The barren tip mutant was controlled by two dominant genes that caused severe yield loss (Meng et al. 2007). *RAMOSA3* (*RA3*) encodes a trehalose-6-phosphate phosphatase, which regulates the inflorescence branching by modification of a sugar signal that moves into the axillary meristems (Satoh-Nagasawa et al. 2006). The Barren stalk fastigiate1 (*Baf1*) gene encodes a putative transcriptional regulator containing an AT-hook DNA binding motif that is required for the formation of maize ears (Gallavotti et al. 2011).

In wheat, *Triticum aestivum* SKP1-related (*TaSKP*) gene family members are involved in the regulation of spike and grain development (Hong et al. 2013). *WFL* (wheat FLO/ LFY) is associated with spikelet formation and also plays an important role in developing the palea in wheat florets (Shitsukawa et al. 2006). S-phase kinase-associated protein 1 (*SKP1*), are component of the SKP1-Cullin-F-box (SCF) E3 ubiquitin ligase complex, functions as an adaptor protein, connecting cullin and F-box proteins. *SKP1* plays crucial roles in cell-cycle progression, transcriptional regulation, flower formation, signal transduction, and many other cellular processes. Green fluorescent protein-tagged *TaSKP* proteins were targeted to the plasma membrane or cytoplasm in plant cells. *TaSKP1*, *TaSKP5*, and *TaSKP6* proteins may act as a bridge between various F-box proteins and cullin proteins and that *TaSKP* genes may be involved in various growth and flower development processes (Hong et al. 2013). However, the genetic mechanism underlying the specification of grass inflorescence and spikelet morphology is still a challenging question for biologists (Zanis 2007).

Development of Spikelet and panicle in Rice

Seed production in rice (*Oryza sativa* L.) largely depends on the number of flowers, which is regulated by the inflorescence architecture. Thus, detailed description of rice inflorescence and spikelet development would be valuable for characterizing mutant phenotypes. The developmental courses of inflorescence and spikelet, and divided their development into nine and eight stages, respectively (Ikeda et al. 2004).

The beginning of rice inflorescence development, known as panicle initiation (PI), occurs when the shoot apical meristem (SAM) transitions into an inflorescence meristem (IM). Over ~7 days, the IM undergoes several transitions into primary branch meristems (PBMs) before ultimate abortion

(McSteen 2006; Yoshida and Nagato2011). After IM abortion, floret development and elongation of axes occurs overroughly 30 days until heading, the point at which the panicle emerges to begin reproduction (International Plant Genetic Resources Institute and Association 2007). Several investigations have described and categorized inflorescence and spikelet development in rice (Matsushima and Manaka 1956; Takeoka et al. 1992).

In the early stage of rice inflorescence development, after the last foliage leaf emerges, a transition from vegetative to reproductive phase occurs and the shoot apical meristem (SAM) is converted inflorescence meristem (IM) (Ikeda et al. 2004). Rice develops a central inflorescence stem that terminates after the formation of several primary and secondary branches. Spikelets are directly produced on primary and secondary branches that are attached on the main axis called the rachis (Itoh et al. 2005).

In the rice inflorescence development, three major factors, namely, timing of IM abortion, conversion of the rachis branch meristem to the terminal spikelet meristem and programme shift of lateral meristem identity along a basal-apical direction on a rachis branch, determine the overall architecture of the rice panicle (Ikeda-Kawakatsu et al. 2009).

Environmental factors affected panicle architecture

The reproductive stage is the most critical stage for inflorescence architecture during crop growth, because it strongly affects yield and seed quality. Improving crop growth and yield under different growth conditions is thus a major goal of plant breeding. Drought stress negatively affects flower pollination by decreasing the amount of viable pollen grain,

increasing the unattractiveness of flowers to pollinators, and decreasing the amount of nectar produced by flowers (Alqudah et al. 2011). Currently, the best way to ensure optimal panicle development in rice, and thus stable yield performance, is to strictly control irrigation and fertilizer regimens from several weeks before PI begins until grain filling initiates (Chen et al. 2007). Water stress caused high rates of pre-flowering spikelet abortion, and spikelet number was reduced by 48% on average and at the meiosis stage (10-20 days before heading) induced the highest frequency of pre-flowering spikelet abortion (Kato et al. 2008).

Panicle architecture that contributing to yield loss in rice

Spikelet degeneration is one of the most important factors causing yield loss (Kobayasi and Imaki 1997; Senanayake et al. 1994). Many floral meristem identity genes play a key role in floral organ formation. The spikelets abortion reduces the grain number per panicle (Ansari et al. 2003; Sheehy et al. 2001; Yamagishi et al. 2004) which is termed as pre-flowering abortion (Senanayake et al. 1991).

Several distinctive mutations and genes involved in panicle architecture were reported in rice, such as *MONOCULM1 (MOC1)*, which had fewer branches on the panicle (Li et al. 2003); *FRIZZY PANICLE (FZP)*, which suppressed the formation of the axillary meristems of the rice spikelets (Komatsu et al. 2003b); *LAX PANICLE (LAX)*, which controlled the rachis-branches during panicle development (Komatsu et al. 2003a); *Aberrant panicle organization 1 (APO1)*, which was involved in the panicle structure (Ikeda et al. 2007); *aberrant panicle organization 2 (apo2)*, which exhibited small panicles with a reduced number of primary branches (Ikeda-Kawakatsu

et al. 2012); *SP1*, which encoded a putative transporter that affected the panicle size (Li et al.2009); non-panicle mutant (nop), which controlled the initiation of the inflorescence differentiation (Wu et al.2009); protein-containing ATPases have been found to be involved in many fundamental processes such as pollen tube growth (Lucca and Leon 2012), vegetative development, and inflorescence architecture (Wang et al. 2011), and *TAW1*, which regulated the inflorescence architecture in rice through inflorescence meristem activity and suppression of the transition to spikelet meristem identity (Yoshida et al. 2013).

Dwarf mutants in higher plants

Dwarf mutants in plants are plying vital role for elucidating regulatory mechanisms for plant growth and development. The greater grain yields were associated with improved lodging resistance and the resulting ability to tolerate higher rates of chemical fertilizers (Worl and et al. 1994), and also with increased harvest index (Miralles and Slafer 1995). However, short stature also contributes to reduced seedling vigour and coleoptile length and may reduce crop water-use efficiency (Rebetzke et al. 2004; Richards 1992; Botwright et al. 2005), and performance in unfavorable environments (Rebetzke et al.1999; Butler et al. 2005; Chapman et al. 2007).

Plants show different architecture and their controlling genes have been recently categorized into three classes, those affecting hormone metabolism and signaling, transcription and other regulatory factors, and cell cycle regulators (Busov et al. 2008). In recent years, the regulatory mechanisms of plant height have been revealed by studies using dwarfing mutant genes in different cereal crops. In wheat, there are many genes associated with a dwarf and semi-dwarf growth habit in wheat (Elliset al.

2005). The *Rht-B1* and *Rht-D1* genes were shown by Peng et al. (1999) to encode DELLA proteins, transcriptional regulators that act to repress GA signaling (Sun 2010). *Rht-B1* and *Rht-D1* confer "GA-insensitive" dwarfism by producing DELLA proteins (Pearce et al. 2011). Two of these genes, *Rht1* and *Rht3* are allelic at the renamed Rht-B1 locus (Borner et al. 1996; McIntosh et al. 2008). *Rht-B1c*, a dominant *GAI* allele which confers more extreme characteristics is caused by a terminal repeat retrotransposons in miniature insertion in the DELLA domain (Wu et al. 2011). In maize, four genes identified that involved in GA biosynthesis and signaling namely, *an1* (Bensen et al. 1995), dwarf8 and dwarf9 (Lawit et al. 2010) and Dwarf3 (Winkler and Helentjaris 1995).

Hormonal effect on dwarfism

Plant dwarfism, a major phenotypic trait in plant breeding, is caused by defects in various hormones, but only gibberellins and brassinosteroid (BR) have been studied extensively. Several rice mutants defective in gibberellin and BR biosynthesis or perception have been isolated and categorized, revealing the typical characteristics of gibberellins and BR related mutants (Mandava 1988, Clouse and Sasse 1998, Taizand Zeiger 2002, Fujioka and Yokota 2003). For example, gibberellins related rice mutants typically show dwarfism with deep green, rough leaves but exhibit no other abnormal morphology (Sakamoto et al. 2004). In contrast, BR-deficient rice mutants usually show dwarfism with other abnormal morphological traits, including malformed leaves with twisted and stiff blades (Hong et al. 2002, Hong et al. 2004, Hong et al. 2005). Recently, there are some new category of growth regulators were identified that controls cell

growth and plant architecture such as novel putative esterase (Gao et al. 2009), biosynthesis of strigolactones (Arite et al. 2009; Lin et al. 2009).

Mutations in GA-related genes are responsible for the semi dwarf phenotypes associated with the green revolution (Hedden 2003) and dwarf or semi-dwarf phenotypes useful for agronomic improvement (Salas Fernandez et al. 2009). GA-related mutants are categorized into GA-deficient mutants and GA-insensitive mutants according to their responses to exogenous GAs (Mitsunaga et al. 1994). In GA-deficient dwarfs, the mutations are usually ascribed to the deficiency in GA metabolic pathway, where the GA metabolism is blocked or weakened (Hedden and Phillips 2000). The enzymes catalyzing the early steps in the GA biosynthetic pathway (i.e. CPS, KS, KO, and KAO) are mainly encoded by single genes, while those for later steps (i.e. GA20ox, GA3ox, and GA2ox) are encoded by gene families. The remaining CPS like, KS-like, and KO-like genes were likely to be involved in the biosynthesis of diterpene phytoalexins rather than GAs because the expression of two CPS-like and three KS-like genes (OsCPS2, OsCPS4, OsKS4, OsKS7, and OsKS8) were increased by UV irradiation, and four of these genes (OsCPS2, OsCPS4, OsKS4, and OsKS7) were also induced by an elicitor treatment (Sakamoto et al. 2004). Of all, most of the genes encoding enzymes of GA metabolism have been identified in Arabidopsis, rice and other model plants (Sakamoto et al. 2004; Grennan 2006), and the complex pathways of GA metabolism have been elucidated in higher plants (Hedden and Phillips 2000; Sun and Gubler 2004; Yamaguchi 2008). In recent years, the power of molecular genetics has dramatically facilitated our understanding of all aspects of GA signaling, especially the principal steps (fig. 1) associated with GA perception and signal transduction in Arabidopsis and rice (Ueguchi-Tanaka et al. 2007).

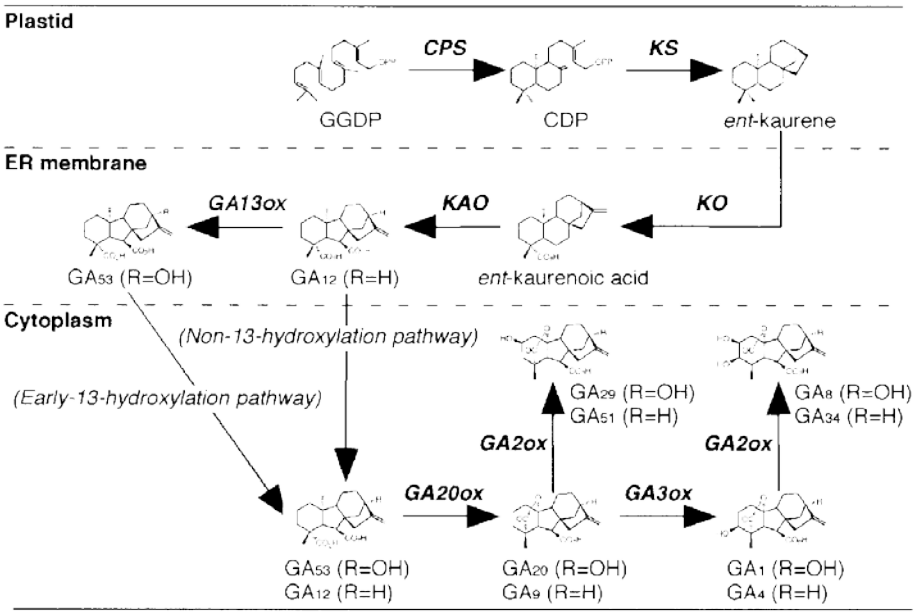


Figure 1. Principal pathway of GA metabolism in plants (Sakamoto et al. 2004)

Dwarfism in rice

In rice, more than 60 dwarf mutants have been reported so far and several of them have been characterized thoroughly (http://www.gramene.org/rice_mutant/). Mitsunaga et al. (1994) classified rice dwarf mutants into three groups: T, D and E. Off these, the T group comprises GA-deficient mutants and the D group GA-insensitive mutants, whereas the E group comprises mutants that are neither GA-deficient nor GA-insensitive. Takeda (1977) categorized rice dwarf mutants into six groups according to elongation patterns of the upper five internodes. In the dn-type mutant, the length of each internode is uniformly reduced resulting in an internode elongation pattern very similar to that of the wild-type. In contrast, the dm-type mutant shows specific reduction of the second

internode. Shortening of a specific internode is also observed in the sh-type mutant, in which only the uppermost internode is shortened. In the d6-type mutant, internodes below the uppermost are shortened. In the nl-type mutant, the fourth internode is relatively longer but the first internode is truncated. However, many rice dwarf mutants also present other phenotypes, such as small grain, more tillers and deformed leaf shapes or plant statures. In terms of these secondary traits, the dwarf mutants could be further defined as small grain dwarf, high tillering dwarf, and malformed dwarf in addition to the typical dwarf and semi dwarf types (Iwata et al.1995), indicating that different mechanisms may be involved in the individual dwarf mutants.

To understand the molecular mechanism of different dwarfism in rice, there are many genes have been reported with different secondary characters together with normal dwarf and semi-dwarf such as high tillering dwarf (Arite et al. 2007; Gao et al. 2009; Lin et al. 2009; Zhang et al. 2011; Zou et al. 2005), less tillering dwarf (Tong et al. 2009), small grain dwarf (Ashikari et al. 1999), slender rice mutant (Ikeda et al. 2001), twisted dwarf (Sunohara et al. 2009) and malformed dwarf (Hong et al. 2003; Yamamuro et al. 2000). Many studies of dwarf mutants in rice have been reported but there are few reports on small grain dwarf mutant even the grain shape plays a major role in consumer preferences in different countries and populations (Weng et al. 2008; Bai et al. 2010; Shao et al. 2010).

In this present study, we study the molecular mechanisms involved in both panicle apical abortion and a small grain dwarf rice mutants through map-based cloning. Thus, our new study will be provided important information in rice breeding and extended the knowledge behind molecular mechanism of dwarfism and panicle apical architecture in rice.

CHAPTER I

Map-based cloning of a panicle apical abortion mutant gene in rice

ABSTRACT

The panicle architecture is an important trait that directly affect grain yield. The rice panicle consists of rachis branches and spikelets, in which panicle apical abortion often occurs. However, little is known about the genetic information available to avoid panicle apical abortion in rice breeding programs. To investigate the mechanism of panicle apical abortion, we characterized a mutant for panicle apical abortion (PAA-Hwa) developed from a *japonica* rice cultivar Hwacheongbyeo treated with N-methyl-N-nitrosourea. Under normal growth conditions, the mutant displayed multiple abnormal phenotypes, such as a slight reduction in plant height, narrow and dark green leaf blades, and small erect panicles with aborted panicle tip compared to the wild-type plants. The presence of panicle apical abortion in this mutant is not resulted from biotic factors. The PAA-Hwa mutant showed clear panicle apical abortion during panicle development before flowering, which severely affected grain yield.

Genetic analysis revealed that the panicle apical abortion was controlled by a single recessive gene, which is tentatively designated as *paa-h*. The *paa-h* gene was fine mapped at an interval of 71 kb flanked by STS markers aptn3 and S6685-1 at the long arm of chromosome 4. Sequence analysis of the candidate genes within the delimited region, *LOC_Os04g56160* gene showed a single base-pair change corresponding to

an amino acid substitution from glycine to glutamic acid. The LOC_*Os04g56160* gene encodes a Plasma membrane ATPase protein containing 951 amino acids. The T-DNA mutant line of *LOC_Os04g56160* gene demonstrated the same phenotype of the original PAA-Hwa mutant, which confirmed the function of *LOC_Os04g56160* gene for the panicle apical abortion phenotype in rice.

Keywords: Rice, panicle apical abortion, Plasma membrane ATPase, Map-based cloning

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important food crops in the world and also staple food for heavily populated Asian countries as well as many African countries. Plant architecture in cereal crops is considered to be a major factor that influences grain yield through the efficient use of solar radiation and optimal partitioning of photosynthates into organs that form grain yield (Wang and Li 2008). Of all of the plant parts, panicles play a key role that contributes directly to grain productivity, and optimal panicle structure, size, and shape has become one of the breeding objectives for higher yield (Sakamoto and Matsuoka 2004).

Plants undergo a series of complicated events in order to form the specific panicle structure during development. All inflorescence structures are generated from inflorescence meristems, which are defined by the WUS-CLV feedback regulatory loop in arabidopsis (Schoof et al. 2000). In the early stage of rice inflorescence development, after the last foliage leaf emerges, a transition from vegetative to reproductive phase occurs and the shoot apical meristem (SAM) is converted inflorescence meristem (Ikeda et al. 2004). The timing of meristem phase change is crucial in the control of inflorescence architecture in grass species (McSteen et al. 2000; Bortiri and Hake 2007; Wang and Li 2008). In recent years, the regulatory mechanisms of panicle development have been revealed by different studies using mutant panicle genes in different cereal crops. In maize, some mutant and genes are reported to be regulatory mechanisms. The barren tip mutant was controlled by two dominant genes that caused severe yield loss (Meng et al. 2007). *RAMOSA3 (RA3)* encodes a trehalose-6-phosphate phosphatase, which regulates the inflorescence branching by modification of a sugar signal that

moves into the axillary meristems (Satoh-Nagasawa et al. 2006). The *Barren stalk fastigiate1 (Baf1)* gene encodes a putative transcriptional regulator containing an AT-hook DNA binding motif that is required for the formation of maize ears (Gallavotti et al. 2011). In wheat, *Triticum aestivumSKP1*-related (*TaSKP*) gene family members are involved in the regulation of spike and grain development (Hong et al. 2013). *WFL* (wheat *FLO/ LFY*) is associated with spikelet formation and also plays an important role in developing the palea in wheat florets (Shitsukawa et al. 2006).

The inflorescence architecture is primarily determined by the basic pattern of the floral branches and the position of the flowers (Coen and Nugent 1994), and this feature greatly influences grain yield. In rice inflorescence, three important factors, namely, inflorescence meristem abortion time, rachis branch meristem change to the terminal spikelet meristem, and the shift to a lateral meristem identity along a basal-apical direction on a rachis branch, determine the general panicle architecture (Ikeda-Kawakatsu et al. 2009). Several distinctive mutations and genes involved in panicle architecture were reported in rice, such as *MONOCULM1 (MOC1)*, which had fewer branches on the panicle (Li et al. 2003); *FRIZZY PANICLE (FZP)*, which suppressed the formation of the axillary meristems of the rice spikelets (Komatsu et al. 2003b); *LAX PANICLE (LAX)*, which controlled the rachis-branches during panicle development (Komatsu et al. 2003a); *Aberrant panicle organization 1 (APO1)*, which was involved in the panicle structure (Ikeda et al. 2007); *aberrant panicle organization 2 (apo2)*, which exhibited small panicles with a reduced number of primary branches (Ikeda-Kawakatsu et al. 2012); *SP1*, which encoded a putative transporter that affected the panicle size (Li et al. 2009); non-panicle mutant (nop), which controlled the initiation of the

inflorescence differentiation (Wu et al. 2009); and *TAW1*, which regulated the inflorescence architecture in rice through inflorescence meristem activity and suppression of the transition to spikelet meristem identity (Yoshida et al. 2013). Cytokinin was also reported to be involved in the inflorescence architecture through the vascular system (Ashikari et al. 2005).

In a practical viewpoint, spikelet degeneration is one of the most important factors causing yield loss (Kobayasi and Imaki 1997; Senanayake et al. 1994), in which the degenerated florets were affected both by genetic (Gonzalez et al. 2005) and/or environmental factors (Gonzalez et al. 2011). Many floral meristem identity genes play a key role in floral organ formation. The spikelets abortion reduces the grain number per panicle (Ansari et al. 2003; Sheehy et al. 2001; Yamagishi et al. 2004), which is termed as pre-flowering abortion (Senanayake et al. 1991). Spikelet abortions can take place either in the apical or basal part of the panicle. To date, most of the reported genes affect the basal part of the panicle. The recently reported *SP1* gene influences the panicle size through spikelet abortion on the basal part of the panicle (Li et al. 2009) and the barren tip genes in maize cause severe yield loss (Meng et al. 2007). Cheng et al.(2011) reported that quantitative trait loci have major effects on panicle apical abortion (PAA) in rice. However, there have been very few reports about PAA and also the reason behind the panicle tip formation is still missing.

The elucidation of the genetic mechanisms regulating panicle architecture would enable the exploitation of the inflorescence characteristics for a better yield (Kellogg 2007; Rao et al. 2008) but the PAA often occur that cause yield loss. In rice breeding program, abnormal panicle tip development phenomenon treated as an undesirable trait by breeders.

Therefore, it is necessary to study this event to provide important information for rice breeding and improve rice production.

In this study, we identified a novel panicle apical abortion mutant, PAA-Hwa, through a chemical mutagenesis, characterized the mutant's effects on some agronomic traits, and isolated the candidate gene through a map-based cloning approach.

MATERIALS AND METHODS

Plant materials and growth conditions

The PAA-Hwa mutant was induced by chemical mutagenesis of a *japonica* rice cultivar Hwacheongbyeo using N-methyl-N-nitrosourea treatment, and the mutant line was genetically fixed with successive generation advancement. The seeds of the PAA-Hwa mutant were taken from the M_{12} generation for this study. For the genetic analysis and fine mapping of the *paa-h* gene, we constructed two F_2 populations from the crosses with Hwacheongbyeo and Milyang23. All the agronomic traits were evaluated during the developmental stage at the experimental farm of Seoul National University, Suwon, South Korea in the summer season of 2010 and 2011. More than ten plants of each parental cultivar were averaged and used as the measurements for each plant.

Genetic analysis

For genetic analysis of the PAA-Hwa mutant, two F_2 populations were constructed from the crosses between mutant and two wild type varieties, Hwacheongbyeo, which was the background of the PAA-Hwa mutant and Milyang23 (Tongil type rice, derived from an *indica*×*japonica* cross and similar to *indica*). The numbers of aborted and normal panicle plants in both of the F_2 populations were counted after heading. Chi-square tests were carried out to determine the fitness ratio of 3:1 in the F_2 populations.

Fine mapping of the *paa-h* locus

Rice genomic DNA was extracted from the fresh leaves of the F_2 plants using the method of Woo et al. (2008). Bulked segregant analysis (BSA) was used to identify the candidate markers linked to the *paa-h* locus. Two contrasting bulks were prepared from the F_2 populations of mutant and milyang23, and each of the bulks (mutant and wild type) contained DNA from eight plants that were pooled into a single sample (Michelmore et al. 1991). Linked markers were confirmed by recessive class-class analysis (Zhang et al. 1994) for each individual within the bulks. The genetic linkage between the *paa-h* locus and molecular markers were determined by PCR (5 min at 94 ^{0}C, followed by 35 cycles of 30 sec at 94 ^{0}C, 30 sec at 55-59 ^{0}C, 30 sec at 72 ^{0}C and final extension of 7 min at 72 ^{0}C) using the linked sequence tagged site (STS) markers (Table 1-1) developed by Chin et al. (2007) in the Crop Molecular Breeding Lab, Seoul National University, Seoul, Korea. Additional STS markers for fine mapping were developed based on the Nipponbare genome sequence (http://rgp.dna.affrc.go.jp/blast/runblast.html). The amplified PCR products were separated on 2.5 % agarose gel containing 0.1 μg/ml ethidium bromide and o.5X TBE running buffer.

Identification of the mutation sites in the PAA-Hwa mutant

To identify the mutation point of the *paa-h*, the candidate genes were sequenced by dividing them into small overlapping fragments. These fragments were amplified using genomic DNA extracted from the PAA-Hwa mutant and wild-type plants using specific primers for each fragment. The amplification products were purified using a PCR purification kit (iNtRON,

Biotechnology, Korea) for TA cloning. The amplified DNA fragments were introduced into pGEM-T Easy Vector (Promega, USA) and the transformed into the *E. coli* strain DH5α. The obtained sequences were compared with the expected sequences by means of CodonCode Aligner software (version 1.6.3; CodonCode Corporation). Sequence alignment was performed with the BLAST network services in National Center for Biotechnology Information (NCBI) and the European Bioinformatics Institute (EBI, www.ebi.ac.uk).

Table 1-1. The PCR-based molecular markers used in fine mapping of the *paa-h* gene

STS marker	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Originated clone	Size (bp)
40100-7	GGATAGGGCCATAGGGGATA	AGCAATCAGCTGGAACCTGT	AL607004	196
40102.1-1	CCGGTCCATATGATCAGTCC	GGGAAACGTGCAGTACCACT	AL662996	197
S04113	GGCATTTAGGTTTGGGGATT	GAATGCATGTTTAGATCCACGA	AL606454	155
S6635	CAAGGTTTCTGCACAAGCTG	TGTGTATGAGGGTTGGTGGA	AL606635	222
S6692	AACCAGTCACTGTTGGCTGA	TTCTTGTTCAGCGGTTTGTG	AL606692	224
S6690	CTTCGCTCCTTTCCTTTCCT	TCAAGTACCGGATTGAGTTTGA	AL606692	238
S6446-2	GAAGCTCCCAAACAGTCGAA	CAAACGCCAATGCTTTGTT	AL606446	286
S6446-3	CTGCACTTGCTGTTCCATCT	CGCCAACTAATCCCAAGCTA	AL606446	270
aptn8	CAATGGCGGAGGTAGGTGTA	CACGCAAAATTAACCCCATT	AL606446	236
aptn3	GGCACTGTTTGCCCATACTAA	AAACGTAGCGGAGCTTTTGA	AL606446	152
S6685-1	CCAGTTCCCAAGTTGCCTTA	TTCCGGATGACTTGTTTCCT	AL606685	199
S2953	ACGGAAAACCTTCTCGCATT	CCTCGTGGTATCCGATTCTG	AL662953	157
S6608	CATATGCATGCCCTTGATGA	AAAAATCAAGGAGCCCCAAC	AL662953	228
S04120	GGTTGAGGCTTAGCAAGGTG	TCGACTCTCTCAGCGTGATG	AL606999	156

dCAPS marker analysis

For the dCAPS analysis of the *paa-h* allele, primers (forward, 5'-CTCGTGTACATCAGATTTATGCAGT-3'; reverse, 5'-CCAGATCAGAGCAATAAGCAAGAAT- 3') were designed using the Web server dCAPS Finder 2.0 program (http://helix.wustl.edu/dcaps/dcaps.html). These primers produced a 160-bp PCR product with an EcoRI site specifically in the wild type. Each product (5 µl) was digested with EcoRI in a total volume of 10 µl and incubated at 37^0C for 1 h. After digestion, the total volume of each digest was separated on a 2.5 % agarose gel and then visualized under UV light.

RESULTS

Phenotype of the PAA-Hwa mutant

The mutant used in this study was isolated by MNU treatment of the *japonica* rice cultivar Hwacheongbyeo. Some of the agronomic traits of the mutant were measured in comparison to the background wild-type parent, Hwacheongbyeo. At the seedling stage, the mutant showed a relatively weak appearance with poor rooting ability, narrow leaves, and discoloration in the leaf tip blades (Fig. 1-1a). The mutant displayed narrow and dark green leaves from the tillering to the reproductive stage in comparison to the wild-type plants (Fig. 1-1b, c, e). The mutant also showed a slight reduction in plant height, evenly shortened internodes, drastically reduced spikelet number per panicle, reduced spikelet fertility, smaller grain shape, and smaller erect panicles with clearly aborted panicle tips (Fig. 1-1c, d, f, g; Table 1-2). At the early panicle development stage (12 days before heading, DBH), there was no significant difference observed, but the PAAs began at 9 DBH and remained until maturity (Fig. 1-2a–g). However, the basal part of the panicle showed a normal spikelet structure (Fig. 1-2h). This result suggests that the PAA-Hwa mutant showed several morphological defects in the plant architecture, especially in panicle and that PAA starts after panicle initiation.

Table 1-2. Agronomical traits of the PAA-Hwa mutant and wild-type cultivar Hwacheongbyeo

Trait	HD	CL (cm)	PL (cm)	PN (No)	SN (No)	SF (%)	1000 seed wt (g)	GL (mm)	GW (mm)	GS
Mutant	Aug20	72.3	14.8	14.6	40	68.4	21.5	5.4	3.2	1.68
Wild type	Aug19	85.6	19.6	13.3	117	90.8	25.1	5.9	3.3	1.79
Difference	NS	*	**	NS	**	**	**	**	NS	*

HD Heading date CL Culm length PL Panicle length PN Panicle number per hill SN Spikelet number per plant SF Spikelet fertility 1000 seed wt (weight) GL Grain length GW Grain width GS Grain shape (length/width)

*, ** significant at 5 and 1% level of probability and ^{NS} represented non-significant difference, respectively.

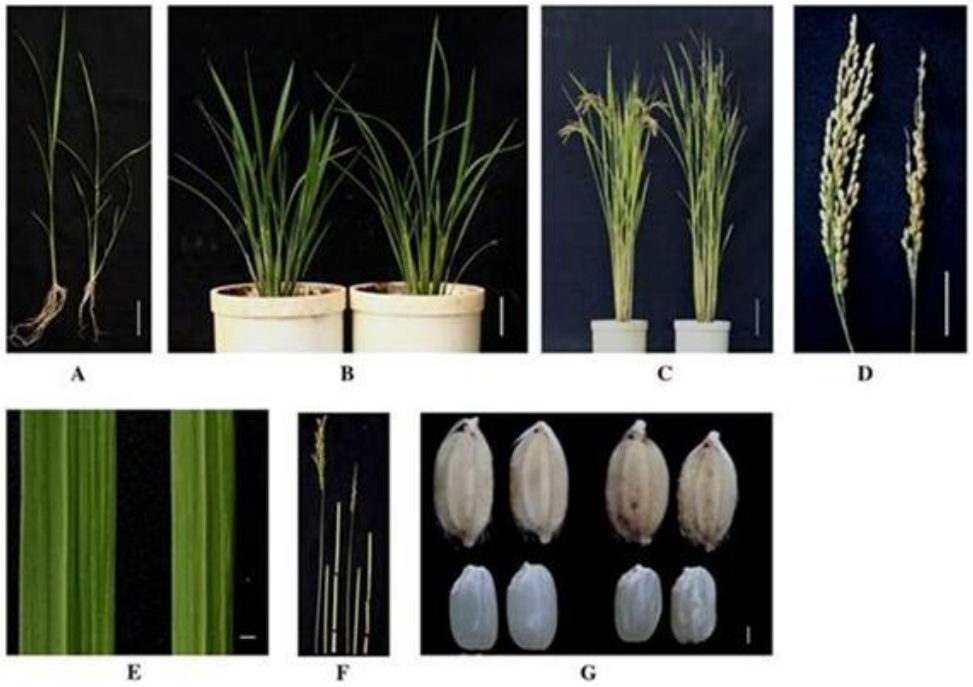


Figure 1-1. Phenotypic characterizations of the wild-type (left) and PAA-Hwa mutant (right); **a** Seedling stage, **b** tillering stage, **c** ripening stage, **d** panicle at maturity stage, **e** leaf blades, **f** internodes elongation pattern, and **g** paddy rice and brown rice. Bars a 15 cm, b, c 20 cm, d 5 cm, e 2 mm, and g 1 mm

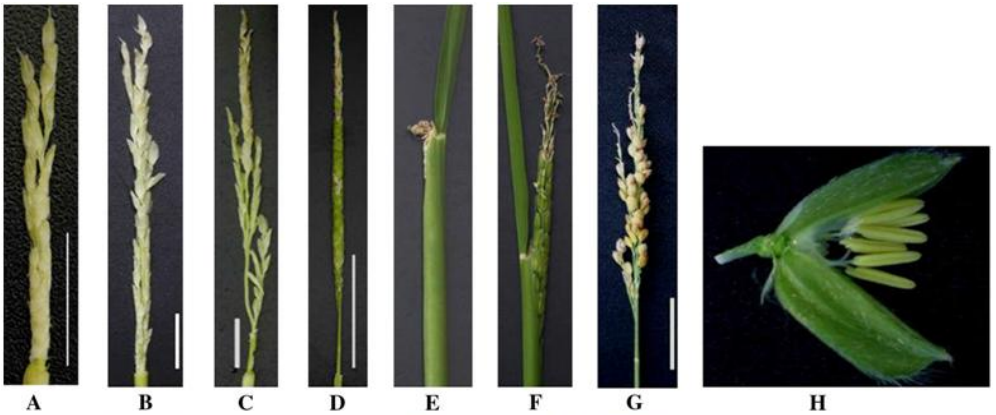


Figure 1-2. Panicle development at different growth stages and spikelet structure for the mutant (top) and wild-type (bottom); **a** 12 DBH (days before heading), **b** 9 DBH, **c** 6 DBH, **d** 3 DBH, **e** flowering initiation, **f** after flowering, **g** ripening stage, and **h** spikelet. Bars a-c 1 cm, and d, g 5 cm

Genetic analysis of the PAA-Hwa mutant

The PAA was determined by the length of aborted panicle tip compare to the total length of panicle. For genetic analysis of the PAA-Hwa mutant, two F_2 populations were developed from the crosses between the mutant and wild-type varieties, Hwacheongbyeo and Milyang23, respectively. The mutant phenotype was easy to distinguish because of the clear aborted panicle tips. In the F_1 progeny, all of the plants displayed normal panicle architecture, and their F_2 progenies displayed a segregation ratio of 3:1 between the wild-type and mutant plants (Table 1-3). The distribution of the aborted panicle tip length inthe mutant plants (Fig. 1-3a) and different degrees of aborted panicle tip for the segregating of 166 F_2 progeny is shown in Fig. 1-3b. Moreover, the F_2 population could be divided into normal (74.1 %) similar to wild type and aborted panicle (25.9 %) similar to PAA-Hwa mutant. It was therefore indicated that PAA is governed by a single recessive gene in rice.

Table 1-3. Genetic segregation of the PAA-Hwa mutant in the two F_1 and F_2 populations

Cross combination	F_1 plants			F_2 plants			$\chi^2(3:1)$
	Total	WT	mutant	Total	WT	mutant	
PAA-Hwa/hwacheongbyeo	13	13	0	289	219	70	0.09
PAA-Hwa/Milyang23	28	28	0	706	540	166	0.83

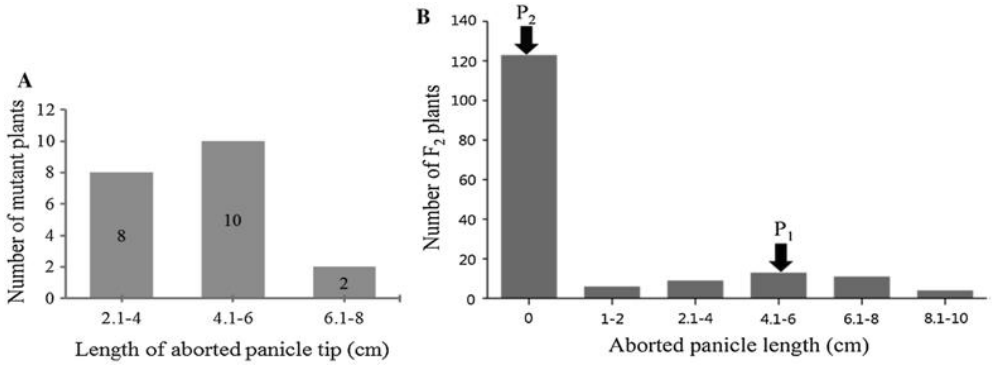


Figure1-3. Distribution of the length of aborted panicle tip in mutant plants **(a)** and in an F_2 population from the cross between PAA-Hwa mutant and Milyang23 **(b)**

Fine mapping of the *paa-h* gene

To elucidate the molecular mechanism of aborted panicle tip, the *paa-h* gene was isolated by map-based cloning. In order to determine the chromosomal location ofthe *paa-h* locus, BSA was performed using F_2 populations from the mutant/Milyang23 cross with 96 polymorphic STS markers distributed across 12 rice chromosomes (Michelmore et al. 1991). Two STS markers (40100-7 and S04120) belonging to chromosome4 were found to be linked to the *paa-h* gene. Thereafter, several markers around 40100-7 and S04120 were used to genotype all of the F_2 plants. Primarily, the *paa-h* gene was mapped at the long arm of rice chromosome 4 between the 40102.1-1 and S04120 markers (Fig. 1-4a). To further narrow down the *paa-h* locus, we developed eleven additional markers according to the genome sequences and the recombinant plants were surveyed by using the new markers. For the aptn3 marker, three recombinants were found. Finally, the locus was fine mapped at an interval of 71 kb an area flanked by new STS markers aptn3 and S6685-1, which corresponded to the sequences on the Nipponbare BAC clones, AL606446 and AL606685 (Fig. 1-4b, c).

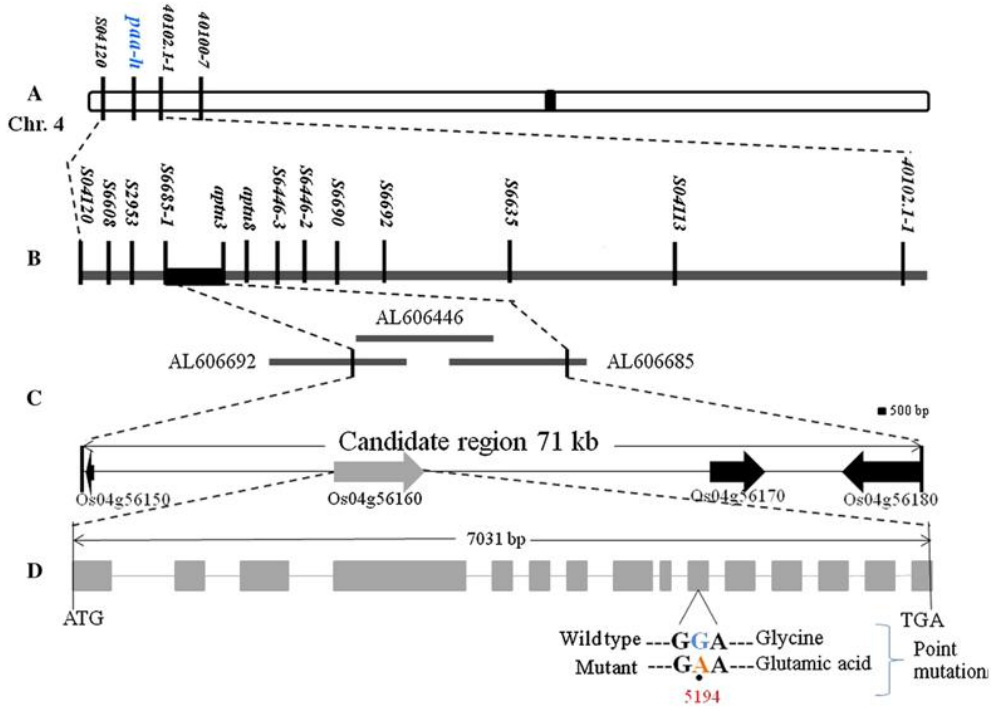


Figure 1-4. Map-based cloning of the *paa-h* gene; **a** Primary mapping of the *paa-h* locus on chromosome 4, **b** the *paa-h* locus was fine mapped with the adjacent markers that are indicated, and **c** identification of the *paa-h* among the candidate genes. Bar 500 bp. **d** The structure of the *paa-h* gene and the mutation site, respectively

LOC_Os04g56160 is the candidate of the *paa-h* gene

Based on the available sequence annotation databases (http://www.gramene.org), there were four predicted genes in this region; *LOC_Os04g56150*, *LOC_Os04g56160*, *LOC_Os04g56170*, and *LOC_Os04g56180*. Among the candidates, *Os04g56150* encodes a putative transcription factor that plays an important role in the control of flower and seed development (Okamuro et al. 1997), and *Os04g56170* encodes squamosa promoter-binding-like protein family, *Os-SPL14* and *OsSPL16* which are associated with panicle branching (Miura et al. 2010) and grain shape (Wanget al. 2012) and *Os04g56180* encodes peroxidase precursor, respectively. A comparison of the genomic DNA sequences within the candidate regions of the PAA-Hwa mutant with those of the wild-type Hwacheongbyeo parent plants as well as the publicly available genome sequence of the cv. Nipponbare indicated the presence of a G/A substitution in the coding region of the *LOC_Os04g56160*. There was no sequence difference detected in the other predicted genes. However, the *LOC_Os04g56160* gene was comprised of 15 exons and 14 introns, and the G/A substitution was predicted to result in a change from glycine to glutamic acid (Fig. 1-4d). The complementary DNA for this gene was 2,856 bp long, and the deduced protein sequence consisted of 951amino acids. Therefore, the amino acid substitution at this position was considered to be responsible for the PAA phenotype in rice.

We further verified the single nucleotide change with a dCAPS marker and screened the F_2 mapping populations. The F_2 population from the cross between the PAA-Hwa mutant and Hwacheongbyeo (wild type) was also screened with the dCAPS marker. We observed the complete co-

segregation of the dCAPS genotypes with the matching phenotypes in the F_2 populations from the cross of PAA-Hwa mutant and Hwacheongbyeo (Fig. 1-5a). In order to determine the distribution of this nucleotide substitution (G/A) among other cultivated rice varieties from the *japonica* and *indica* groups (Table 4), the nucleotide at this position in all of the varieties except the PAA-Hwa mutant was guanine (G). This observation indicated that glycine at position 659 of *LOC_Os04g56160* was consistent in all of the tested rice varieties, which possessed a normal panicle structure (Fig. 1-5b). This result indicates that *LOC_Os04g56160* is a strong candidate gene responsible for PAA in rice.

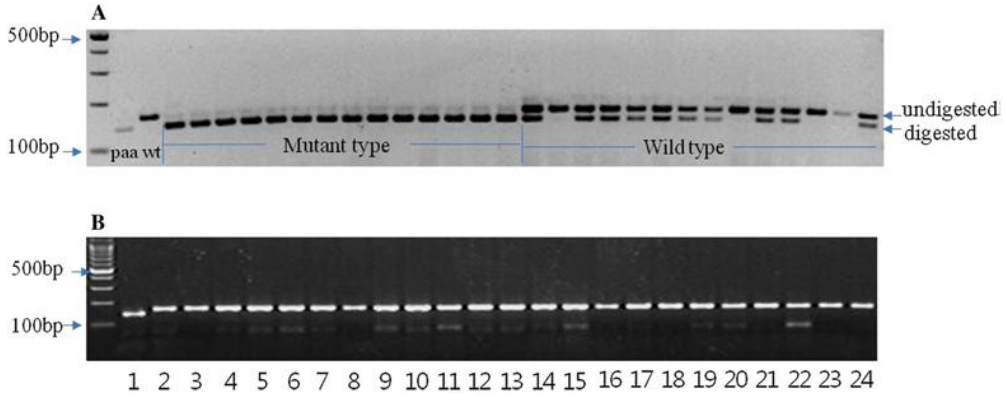


Figure1-5. The dCAPS analysis of the nucleotide substitution (G/A); **a** Co-segregation analysis in the F_2 populations from the PAA-Hwa mutant and the Hwacheongbyeo cross and **b** genotype of the 23 cultivars having normal panicle architecture. Cultivar code and name were as shown in Table 4

Table 1-4. Cultivated rice varieties used in the dCAPS marker analysis

Nos.	Variety names	Origin	Amino acids	Nos.	Variety name	Origin	Amino acids
1	paa mutant	Korea	Glu	13	Chucheong	Korea	Gly
2	Hwacheongbyeo	Korea	Gly	14	Dongjinbyeo	Korea	Gly
3	Milyang 23	Korea	Gly	15	IR36	Philippines	Gly
4	Ilpum	Korea	Gly	16	IR56	Philippines	Gly
5	Sindongjin	Korea	Gly	17	IR64	Philippines	Gly
6	Gopumbyeo	Korea	Gly	18	IR72	Philippines	Gly
7	Odae	Korea	Gly	19	Dasanbyeo	Korea	Gly
8	Ungwang	Korea	Gly	20	Cheongcheong	Korea	Gly
9	Koshihikari	Japan	Gly	21	Shiokari	Japan	Gly
10	Shennong 27	China	Gly	22	Hanmaeum	Korea	Gly
11	Hitomebone	Japan	Gly	23	Hangangchal	Korea	Gly
12	Samgwang	Korea	Gly	24	Bosukchal	Korea	Gly

Analysis of *paa-h* and its homologous protein

To find the probable function of the *paa-h* protein, its sequence was searched in public databases but, surprisingly, it didn't match any known protein. BLAST searches, in which proteins of more than 90 % identity was used for multiple amino acid sequence alignment, revealed that the amino acid sequence of the *LOC_Os04g56160* protein had several putative homologs from *Aeluropus littoralis, Arabidopsis thaliana, Brachypodium distachyon, Capsella rubella, Cucumis sativus, Daucus carota, Glycine max, Hordeum vulgare, Kosteletzkya virginica, Lilium longiflorum, Lupinus albus, Medicago truncatula, Mesembryanthemum crystallinum, Musa balbisiana, Nicotiana plumbaginifolia, Nicotiana tabacum, Olimarabidopsis pumila, Oryza sativa, Phaseolus vulgaris, Picea sitchensis, Populus trichocarpa, Prunus persica, Ricinus communis, Sesbania rostrata, Solanum lycopersicum, Solanum tuberosum, Sorghum bicolor, Triticum, aestivum, Vicia faba, Vitis vinifera, Zeamays*, and *Zosteramarina* (Fig. 1-6). In addition, glycine at the mutant locus was well conserved across plant species, suggesting that the change of glycine to glutamic acid should be the cause of the mutation although its biological function remains unidentified.

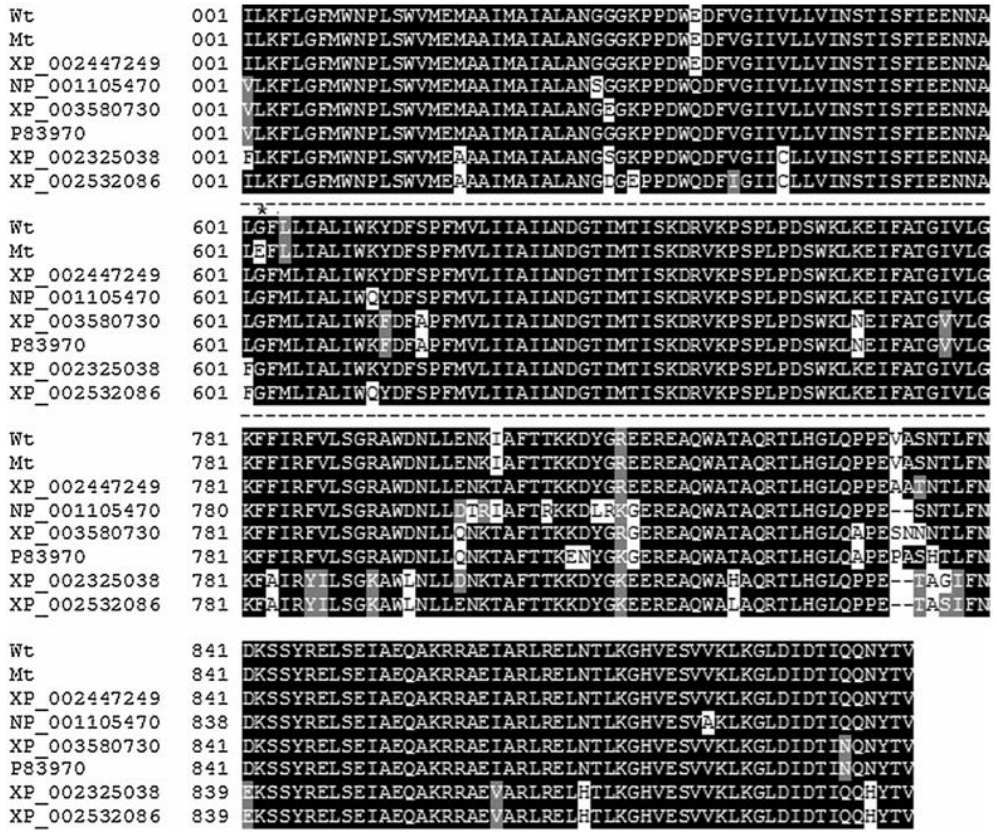


Figure1-6. Alignment of the amino acid sequences of *paa-h* with proteins from *Sorghum bicolor* (accession number XP_002447249), *Brachypodium distachyon* (XP_003580730), *Zea mays* (NP_001105470), *Triticum aestivum* (P83970), *Populus trichocarpa* (XP_002325038), *Ricinus communis* (XP_002532086). Amino acid residues in white on a black background indicate the similar nucleotides. The asterisk indicates the well conserved glycine across plant species.

Characterizations of a T-DNA inserted mutant

To confirm the function of *LOC_Os04g56160* control the panicle apical abortion in rice, we obtained three T-DNA tagging line (3A-06739, 2C-50175 and 2D-31273) from POSTECH (http://signal.salk.edu/cgi-bin/RiceGE and/ http://www.postech.ac.kr). The T-DNA inserted into the first intron, 8thexon and 3´ UTR of *LOC_Os04g56160* (fig. 1-7a). The mutation was confirmed using the different specific primer combinations of three T-DNA lines. A total of 10 seeds from 3A-06739, 10 seeds from 2C-50175 and 16 seeds from 2D-31273 were planted in the growth chamber. Off all T-DNA tagging plants; 19 heterozygous, 9 homozygous wild type and 10 homozygous mutants T-DNA tagging lines were identified (fig. 1-7b). But only the homozygous T-DNA tagging mutant plants exhibited the aborted panicle tip with the same phenotype of the original PAA-Hwa mutant (figs. 1-7c, d), which confirmed the function of *LOC_Os04g56160* gene for the panicle apical abortion phenotype in rice.

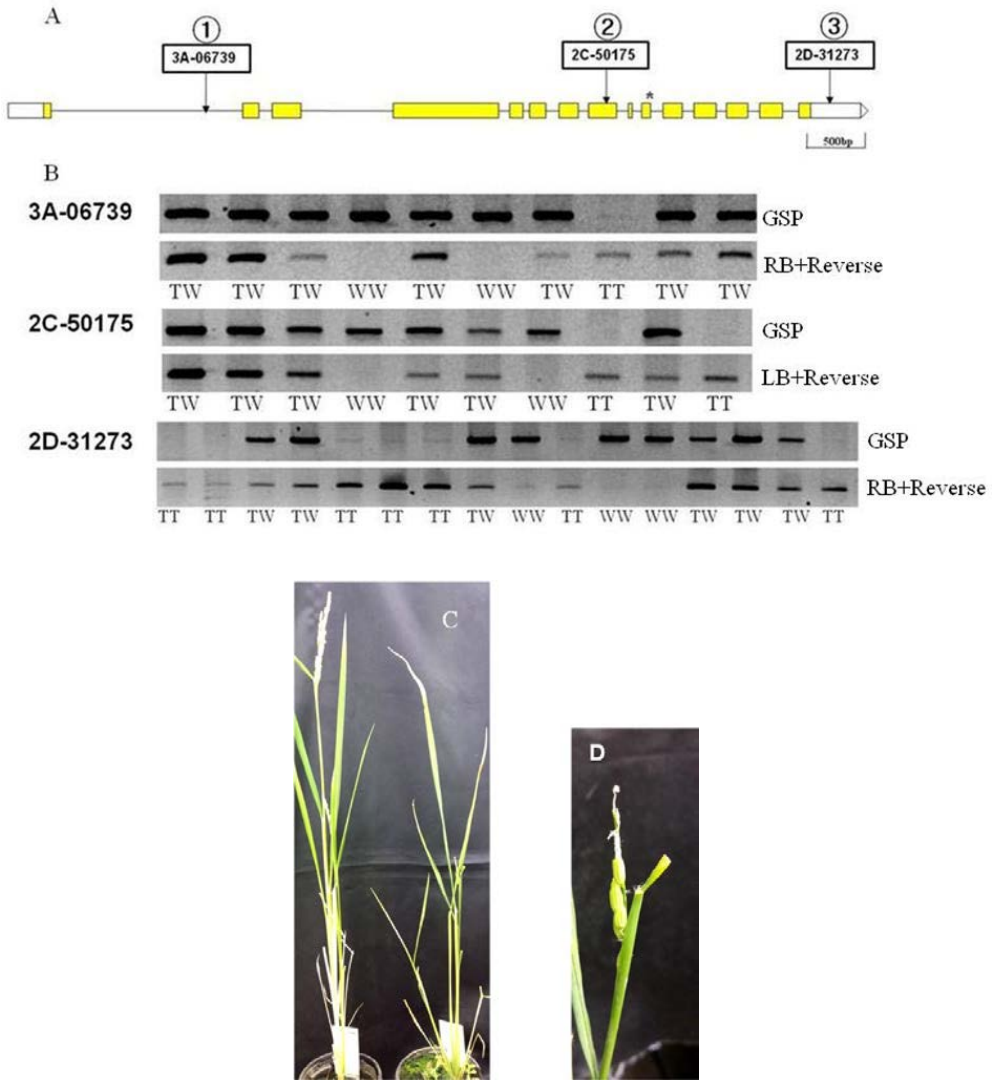


Figure 1-7. Characterizations of a T-DNA inserted mutant; **a** Schematic structure of the T-DNA inserted positions, Exon (yellow boxes) and intron (line between yellow boxes), **b** The PCR bands with specific primer combinations of different T-DNA line, **c** The phenotype of T-DNA inserted plants wild type (left) and homozygous mutant (right), **d** Panicle architecture of the T-DNA homozygous mutant. TT homozygous mutant, WW homozygous wild-type and TW heterozygous

DISCUSSION

Inflorescence architecture is a key agronomic trait, since increasing the number of rachis branches and spikelets as well as overall yield. Inflorescence architecture is mainly depends on the basic pattern of floral branches and the position of flowers (Coen and Nugent, 1994) and there are some unexpected trait occurs during the breeding programs which cause severe yield loss. In this study, we studied a novel rice mutant, PAA-Hwa that exhibits a panicle apical abortion. Under the normal growth condition, the mutant plants displayed significant differences in agronomic traits such as leaf width, plant height, grain length, grain shape, 1,000 seed weight, spikelet fertility, panicle size, and grain yield per plant comparing to the wild type plants. Specially, the mutant showed a distinct panicle character which causes severe yield losses due to the PAA. At the early stage of panicle development (12DBH), there was no significant difference, but the panicle gradually exhibited clear panicle tip abortion after that stage before heading, indicating that the expression of the *paa-h* gene may occur during spikelet development before flowering.

Understanding the genetic mechanism underlying the inflorescence architecture is practically important for developing high-yield varieties in cereal breeding programs. It is known that the inflorescence meristem produces several primary branch meristems before the ultimate abortion. The primaries produce secondaries, which eventually produce spikelets (Kellogg 2007). Spikelet degeneration affects the panicle structure which can occur at either the apical or basal position ofthe panicle. Several genes including *SP1, APO1, MOC1, LAX, RCN1, FZP, FON1,* and *FON4* have been identified as being associated with panicle architecture in rice and also responsible for

causing yield loss (Ashikari et al. 2005; Chu et al. 2006; Komatsu et al. 2003a; Li et al. 2003, 2009; Moon et al. 2006; Nakagawa et al. 2002). However, most of the identified genes affecting panicle architecture are involved in the basal degeneration of spikelets and/or rachis branches. Recently, Li et al. (2009) reported on the cloning of the pre-flowering floret abortion gene *SP1*, which reduces the number of grains per panicle. The *qPAA8* gene was reported to control PAA through the accumulation of excess hydrogen peroxide in rice (Cheng et al. 2011). But the qPAA8 phenotype was dependent on heading date and/or temperature, the degree of abortion was different in case of different temperature or heading date, while very little know about the molecular basis of panicle apical abortion. However, the PAA-Hwa mutant in this study exhibited unique morphological characteristics as a new type of mutant.

Genetic segregation analysis indicated that PAA-Hwa phenotype was controlled by a single recessive gene. *The paa-h* gene was fine mapped at an interval of 71 kb flanked by newly developed STS markers aptn3 and S6685-1. Sequencing of the candidate genes at the locus resulted in the identification of a 1-bp substitution in the gene *LOC_Os04g56160*. This mutation was also confirmed by the dCAPS marker analysis and the analysis of homologs across plant species, indicating that the *LOC_Os04g56160* is a strong candidate gene for the panicle apical abortion of PAA-Hwa mutant.

The *paa-h* gene was predicted to possess the E1-E2 ATPase (also known as P-ATPase) domain-containing protein, which was first identified as using ATP to transport Na^+ and K^+ ions across the axonal membrane (Skou 1957). The plasma membrane proton ATPase is thought to be essential for diverse cellular processes, including nutrient transport, cellular expansion,

physiological processes, and osmoregulation (Morsomme and Boutry 2000; Palmgren 2001). It is expected that fungal and plant plasma membrane ATPases are regulated by protein kinases, because this is the most frequently found regulatory mechanism in eukaryotes (Hunter 1987). Recently, protein-containing ATPases have been found to be involved in many fundamental processes such as pollen tube growth (Lucca and Leon 2012), vegetative development, and inflorescence architecture (Wang et al. 2011).

Finally, our T-DNA inserted mutant line of *LOC_Os04g56160* gene exhibited the same phenotype of the original PAA-Hwa mutant, which confirmed the function of *LOC_Os04g56160* gene for the panicle apical abortion phenotype in rice. Further studies on the *paa-h* gene may provide an opportunity to investigate the molecular regulatory mechanism of the PAA in rice.

CHAPTER II

Characterizations and map-based cloning of a new dwarfing mutant gene in rice

ABSTRACT

Plant height is one of the most important agronomic traits in crop breeding because dwarf cultivars are more resistant to lodging and led to the remarkable yield increases in monocot plants, especially in rice. More than 60 dwarf mutants in rice have been identified up to now and several of them have been well characterized. However, the genetic mechanisms controlling dwarfism are not well characterized, and causal genes underlying most dwarf mutants are still uncovered. We characterized a dwarf rice mutant (dwaf13) derived from Shiokari by multiple rounds of back-crossings.

Phenotypic analysis under the field growth conditions indicated that the mutant had shown multiple altered phenotypes, such as plant height, increased tiller number, short and rounded leaf tip, yellowish stripe on leaf blade, late and asynchronous heading, strong root systems, short erect panicle, smaller seed size, etc. The mutant was categorized as dn-type dwarf mutant according to the elongation pattern of the internodes by Takeda. In addition, the dwarfing of the dwarf13 mutant was responsive to GA based on the analyses of two GA-mediated processes belongs to the group E by Mitsunaga. The expression levels of gibberellin (GA) biosynthetic genes including OsCPS1, OsKS1, OsKO1, OsKAO, OsGA20ox2, OsGA2ox3 and OsGA2ox1 were significantly increased in the dwarf13 mutant. Anatomical

observations revealed that the dwarf13 mutant had less number of vascular bundles and reduced cell size than wild type plants, elucidation the dwarf phenotype.

Genetic analysis indicated that the dwarf13 mutant was controlled by a single recessive gene. By fine-mapping strategy, *d13* gene was flanked by two STS markers namely, DMR-3 and S5789 within the physical distance of 265 kb around the centromeric region of chromosome 9. Based on available sequence annotation databases, there are thirty predicted genes annotated in this region. Further studies of the *d13* gene will be provided a new molecular mechanism of dwarfism related to GA pathways in rice.

Keywords: Rice, Dwarfism, Map-based cloning, Gibberellin (GA)

INTRODUCTION

Dwarf mutants in plants are plying vital role for elucidating regulatory mechanism for plant growth and development. Accordingly, improving crop productivity by selecting for grain yield and optimal plant architecture has been the important factors of rice breeding programs (Wang and Li 2008). The Green Revolution gene (*SD1*), is a striking example of how breeding for changes in rice plant architecture can significantly improve yield potential (Hedden 2003; Asano et al. 2009). The genes involved in dwarfism and semi-dwarfism affect various physiological and biochemical properties and therefore cloning and functional analyses may provide to utilize them in high yielding rice production (Gao et al. 2005; Ma et al. 2009) to fulfill the demand of the increasing world populations (Brown and Funk 2008).

Dwarf mutants have been isolated in many species and have been extensively studied for their mode of inheritance and their response to plant hormones. Plants show different architecture and their controlling genes have been recently categorized into three classes that affected the hormone metabolism and signaling, transcription and other regulatory factors, and cell cycle regulators (Busov et al. 2008). Among the plant hormones, Gibberellin and Brassinosteroid are extensively studied that are responsible for dwarfism in plants (Fujioka and Yokota 2003; Yamaguchi 2008). Off all the hormones, GA_3 which consists of a large family active plant growth regulators that control various developmental and biological processes including seed germination, stem elongation, leaf expansion, trichome development, pollen maturation, induction of flowering and fruit development (Olszewski et al. 2002). Recently, a novel function of GA in regulating cell production has

also been discovered (Achard et al. 2009; Ubeda-Tomás et al. 2009). Besides there are some new category of growth regulators were identified that controls cell growth and plant architecture such as novel putative esterase (Gao et al. 2009), biosynthesis of strigolactones (Arite et al. 2009; Jiang et al. 2013; Lin et al. 2009) and it's levels are mediated by the auxin signal transduction pathway (Beveridge et al. 2010).

In recent years, the regulatory mechanisms of plant height have been revealed by studies different dwarfing mutant genes in different cereal crops. In wheat, there are many genes associated with a semi-dwarf growth habit in wheat (Elliset al. 2005). The *Rht-B1* and *Rht-D1* genes were shown by Peng et al. (1999) to encode DELLA proteins, transcriptional regulators that act to repress GA signaling (Sun 2010). *Rht-B1* and *Rht-D1* confer "GA-insensitive" dwarfism by producing DELLA proteins (Pearce et al. 2011). Two of these genes, *Rht1*and *Rht3* are allelic at the renamed *Rht-B1* locus (Borner et al. 1996; McIntosh et al. 2008). *Rht-B1c*, a dominant *GAI* allele that confers more extreme characteristics is caused by a terminal repeat retrotransposons in miniature insertion in the DELLA domain (Wu et al. 2011). In maize, four genes identified that involved in GA biosynthesis and signaling namely, *an1* (Bensen et al.1995), dwarf8 and dwarf9 (Lawit et al. 2010) and Dwarf3 (Winkler and Helentjaris 1995).

Rice has a large number of dwarf mutants (Kinoshita 1995). Mutations in GA-related genes are responsible for the semi dwarf phenotypes associated with the green revolution (Hedden 2003) and dwarf or semi-dwarf phenotypes useful for agronomic improvement (Salas Fernandez et al. 2009). Takeda (1977) categorized rice dwarf mutants into six groups according to elongation patterns of the upper five internodes. Mitsunaga et

al. (1994) classified rice dwarf mutants into three groups (T, D and E) based on two GA-mediated processes, namely, elongation of shoots and production of α-amylase activity in endosperms. However, there are mainly two kinds of dwarf and semi-dwarf rice mutants were characterized with different distinguished secondary characters such as high tillering dwarf (Arite et al. 2007; Gao et al. 2009; Lin et al. 2009; Zhang et al. 2011; Zou et al. 2005), less tillering dwarf (Tong et al. 2009), small grain dwarf (Ashikari et al. 1999), slender rice mutant (Ikeda et al. 2001), twisted dwarf (Sunohara et al. 2009), malformed dwarf (Hong et al. 2003; Yamamuro et al. 2000) and sword shape dwarf (Hirochika 2001).

Dwarf crop varieties not only increase the resistance to lodging on account of their short stature, but also produce high yields, with increases in harvest index (Khush 2001). But the extensive use of limited dwarfing sources may cause a 'bottleneck' effect in genetic background for developing a new rice varieties (Hargrove and Cabanilla 1979). The utilization of heterosis and improvement of plant architecture are considered as two substantial components to breed super high-yielding rice hybrids (Wu 2009; Yuan 2003). Thus, their applications have been associated with increased yields, higher fertility, early maturity, seed size and high tillering capacity. Many studies of dwarf mutants in rice have been reported but there are few reports on small grain dwarf mutant even the grain shape plays a major role in consumer preferences in different countries and populations (Weng et al. 2008; Bai et al. 2010; Shao et al. 2010). Besides, the molecular mechanisms of this GA-insensitive growth response still remain unclear.

In this present study, we characterized a small grain rice dwarf mutant that is related GA and it will extend the knowledge behind molecular mechanism of dwarfism in rice.

MATERIALS AND METHODS

Plant materials

The dwarf13 mutant was derived from Shiokari by multiple rounds of back-crossings, and the resultant recombinant inbred lines Id13 were used in this study. For genetic analysis of the dwarf13 mutant, two F_2 populations were developed from the crosses between the dwarf13 mutant and two wild-type varieties, Shiokari (*japonica*) and Milyang23 (Tongil type rice, derived from an *indica*×*japonica* cross and similar to *indica*), respectively. The F_2 populations were used for mapping from the cross between the mutant and Milyang23. The F_2 plants were classified on the basis of plant height, seed size and leaf appearances. All the plant materials were grown under natural field condition in the experimental field of Seoul National University, Suwon, Korea in 2010 and 2011. Seeds of all the lines were immersed in water for 2 days and were sown in a nursery bed. About One-month-old seedlings were transplanted to the field at 20×30 cm spacing. Results from more than ten plants of each parental cultivar were averaged.

Measurement of shoot elongation with GA_3 application

To investigate the effect of GA on second leaf sheath elongation, the seeds surface of wild type and mutant were sterilized with a 3% NaClO solution for 30 minutes, washed five times with sterile distilled water and then incubated for 2 days in sterile distilled water at 30^0 C. Germinated seeds were placed on 1% agar medium containing various concentrations of GA_3 (10^{-9} -10^{-5}M) and then grown at 30^0C under continuous fluorescent light until emergence of the second leaf sheath. After 7 days of incubation, the length of the second leaf sheaths were measured and compared with standard deviation.

Assay of α-amylase activity

Six embryoless half-seeds (for each plate) from the mutant and wild type were sterilized with a 3% NaClO solution for 30 min and washed five times with sterile distilled water and then placed on 2% agar plates containing 0.2% soluble potato starch, 10 mM sodium acetate and 2 mM $CaCl_2$ at pH 5.3. The 50% of the plates were prepared by adding 1 µM GA_3 to the cold medium and 50% plates were used as a control (without GA). The plates were incubated for 4 days at 30^0 C in darkness and then flooded with iodine solution. The synthesized and secreted α-amylase from the half-seeds was characterized by clear zones around the seeds on the plate, resulting from digestion of the starch by their secreted α-amylases.

Anatomical features of peduncle tissues

Paraffin-embedded peduncle tissue sections were prepared following the methods described by Piao et al. (2009) with slight modifications. Peduncles were harvested at ripening stage. The 1st internodes were cut and fixed in FAA solution (50% ethanol, 5% acetic acid, 3.7% formaldehyde) and stored at 4°C. The fixed internodes were dehydrated by soaking for 2 h each in an ethanol solution series 70, 80, 95% and finally the internodes were soaked in 100% ethanol. The samples were cleared by soaking for 2 h in the clearing solution series consisting of 75% ethanol/25% histoclear, 50% ethanol/50% histoclear, 25% ethanol/75% histoclear, followed by soaking in 100% histoclear solution overnight. For paraffin infiltration, the cleaned samples were soaked for 2 h in the solution series of 75% histoclear/25% paraffin, 50% histoclear/50% paraffin, 25% histoclear/75% paraffin, and finally, the samples were soaked in 100% paraffin 2 times for 2 h at 60^0C.

The infiltrated sample was embedded in a paraffin block and then cut into 6-8 μm sections using a microtome (MICROM Lab, Walldorf, Germany) and mounted on a Superfrost-plus glass slides (Fisher Scientific, Pittsburgh, PA, USA) and dried at 42^0 C for overnight. The sections were then deparaffinized with 100% xylene for 2h followed by hydration by soaking for 2 min each in 50% ethanol/50% xylene, 100% ethanol, and sterile water. The samples were stained with 1% safranin in 30% ethanol for 30 s followed by washing with sterile water two times. Samples were soaked for 2 min each in 30% ethanol, 50% ethanol, 70% ethanol, 85% ethanol, and 95% ethanol. Finally the sections were cleared by soaking twice in 100% xylene for about 30 min and mounted in Canada balsam. The cross sections of peduncle were observed by optical microcopy at 100X magnification.

DNA extraction and molecular marker analysis

Rice genomic DNA was extracted from fresh leaves of rice plants at seedling stage using the CTAB method (Causse et al. 1994). The F_2 plants derived from the cross between dwarf13 and Milyang23 were used for the identification of molecular markers linked to the dwarf gene. Bulked-segregant analysis (BSA) was performed by the same amount of DNA from ten mutant and ten wild type plants derived from the F_2 populations (Michelmore et al. 1991) using polymorphic 64 STS primers which dispersed throughout the rice genome. After BSA, neighboring STS markers developed by Chin et al. (2007) were used to construct the primary map. For fine mapping, 2 SSR and 13 sequence-tagged site (STS) markers (table 2-1) were developed based on the differences in the DNA sequences between the *indica* and *japonica* rice subspecies (http://www.ncbi.nlm.nih.gov/ for *indica* and http://www.rgp.dna.affrc.go.jp/ for *japonica*).

The PCR analysis was performed in a 20 µl total reaction volume containing 50 ng of template DNA, 0.2 µM of each primer, 10mM Tris-HCL (PH 8.3), 50 mM KCL, 0.01% gelatin, 1.5 mM $MGCL_2$, 0.125 mM dNTPs and 1 unit of Taq DNA polymerase. Template DNA was initially denatured at 94 ^{0}C for 5 minutes, followed by 35 cycles of PCR amplification with 30 sec denaturation at 94 ^{0}C, 30 sec of primer annealing at 55-59 ^{0}C (depends on primer) and 30 sec of primer extension at 72 ^{0}C. Finally, 7 min incubation at 72 ^{0}C was programmed to allow completion of primer extension in a PTC220 dual 96-well thermo-cycler (MJ Research, USA). The amplified PCR products were separated in both 3% agarose gel containing 0.1 g/ml ethidium bromide and non-denaturing 6% polyacrylamide gel in 1X TBE running buffer.

Table 2-1. List of molecular markers used in *d13* gene mapping

STS markers	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')	Originated clone	Prime Size (bp)
S0900A	CCAATTCACGGTTTAACAAGG	GCCATGAAGCTTCGTTAGGA	AP006058	207
S5909	TAAAAACCGGGATGCGACTA	GATTTGATGGGTTGGAGTGG	AP005909	269
R5821	TGCATAGCATATCAACTAGCCCTACC	GCTGAAACAGAATGAAAGCACAGC	AP005821	159
R5787	TCACAGCTTAGTGCATGTTGAGC	GATTCACCTGGCAATGAGAACG	AP005787	190
S5709	TTTGTGTTCAATCCTCCAACC	TTTCTTGCGACACCACAGAG	AP005709	158
DMR-1	GGCGAAAGCCATCTTGAGTA	AAATCAAAAGCGAACCAACG	AP005860	269
DMR-3	TGCATTAAGGACATTCGTGA	CCTGTGCAACGGTTACTAACTT	AP005569	249
S5569-1	CGTAGCATGGTCGTCTTGTG	TTTCCAGCCATATGCAAACA	AP005569	243
S5569-2	GCGCTTGGAGCAAACTAAAC	GCCCATCACACATCTCCAG	AP005569	201
S5789	ATGATCAGGGCATTCACCTC	CCCCACAGCTCAAACTCAAT	AP005789	183
S5560	TGGGCGCTCGAAATTTATAC	GGCCCCTCTCACCATCTACT	AP005560	260
S5587	GGATCTAACGTGGGACATGG	ATCCATCCGTTTCGCATATC	AP005587	201
S5589	TGCGATGTTTCCGCTATTTT	TCTTTCGCTAGGGTTCTCCA	AP005589	202
S5592	ACCATTAGTCGCGGATGAGA	TGGCGCTACAGTGATGCTAC	AP005592	299
S0903.2	GGTCATCACCTTGCCATCTT	TTTCATCATTCTTGCTGGTGA	AP005594	222
S0906.8	TTTGAATGAATCGGCGAACT	TCTGGGACGAGAGAAATCGT	AP005562	240

Sequence analysis of the Candidate genes

To identify the mutation point, genomic DNA sequence of the candidate genes were divided into small overlapping fragments were amplified using genomic DNA extracted from dwarf13 mutant and the background wild type plants by using specific primers for each fragment. The amplified DNA fragments were introduced into pGEM-T Easy Vector (Promega, USA) and the transformed into the *E. coli* strain DH5α and also the amplified PCR products were purified using a PCR purification kit (iNtRON, Biotechnology, Korea) for direct sequencing using specific primer. The obtained sequences were compared with the expected sequences by means of CodonCode Aligner software (version 1.6.3; CodonCode Corporation).

RNA isolation and real-time qPCR

Total RNA was extracted from dwarf13 mutant and wild type plants at tilleing stage, using the TRIzol isolation reagent (Invitrogen, USA) according to the manufacturer's protocol. The RNA was pre-treated with DNase I (Takara, Japan), following the manufacturer's instruction. A total RNA was reverse transcribed using an oligo $(dT)_{15}$ as primers and a M-MLV reverse transcriptase kit (Promega, USA). For real-time qPCR analysis, GA biosynthetic genes including OsCPS1, OsKS1, OsKO1, OsKO2, OsKAO, OsGA20ox2, OsGA2ox3 and OsGA2ox1 primers were used. PCR contained SYBR Premix Ex Taq (Takara, Japan) for the detection of PCR products, and were performed according to the manufacturer's instructions (Bio-Rad, http://www.bio-rad.com/) *actin* used as a reference gene. Three repeats were carried out for each sample. The resulting melting curves were visually

inspected to ensure specificity of product detection, and gene expression was quantified using a C1000 thermal cycler (Bio-Rad, USA).

RESULTS

Phenotypic characterizations of the dwarf13 mutant

Dwarf13 mutant was produced by multiple rounds of back-crossing and the recurrent parent, Shiokari was used in this study. Phenotypic analysis under the field growth condition indicated that the phenotypes of the dwarf13 mutant were comparable with wild type plant from vegetative developmental stage to the maturity stage. At the seedling stage, mutant displayed reduced plant height (Fig. 2-1a). After heading stage, the dwarf13 mutant reached 40–50% of the height of the parental cultivar (Fig. 2-1c). The mutant showed increased tiller number, short and rounded leaf tip, yellowish stripe on leaf blade, late and asynchronous heading, partial male sterility, and stronger roots (figs 2-1 b, c, e & f). The mutant also showed smaller erect panicles, shorter rachis branches and smaller seed size compared to the wild type plants (figs d, g). These observations suggested that dwarf13 is a small grain dwarf mutant and involved in several morphological characters in rice.

Table 2-2. Comparisons of agronomic traits between the d13 mutant and wild-type Shiokari plants

Trait	HD	CL (cm)	PL (cm)	PN (No)	SN (No)	1000 seed wt (g)	GL (mm)	GW (mm)	GS
Mutant	July8	23**	6.7**	18.5**	15.5**	18.2**	4.7**	3.2**	1.5**
Wild type	July6^{ns}	51	12.4	10.4	50.6	25.3	5.8	3.6	1.9

HD Heading date CL Culm length PL Panicle length PN Panicle number per hill SN Spikelet number per plant 1000 seed wt (weight) GL Grain length GW Grain width GS Grain shape (length/width)

**Significant at 1% level of probability and ^{NS} represented non-significant difference

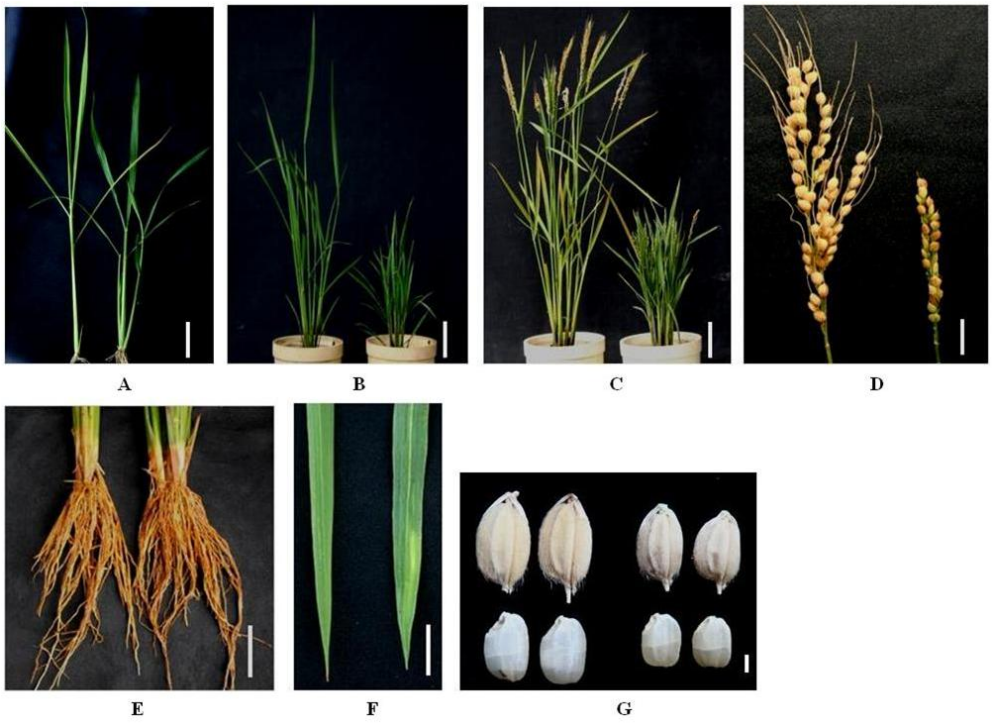


Figure 2-1. Morphological characterizations of the wild type (left) and dwarf13 mutant (right); **a** Seedling stage, **b** Tillering stage, **c** Ripening stage, **d** Panicle at maturity stage, **e** Roots, **f** Leaf tip, and **g** Paddy rice and brown rice. Bars **a** 10 cm; **b,c** 15 cm; **d** 2 cm; **e, f** 5 cm and **g** 1 mm

Internodes elongation pattern

The rice dwarf mutants were categorized into six groups based on elongation patterns of the upper five internodes according to Takeda (1977). All internodes of dwarf13 mutant were shorter than that of wild type plants but 4th (lower most) internode was less affected (Fig. 2-2a). The relative lengths of each internode to the Culm in dwarf13 mutant and wild type (Shiokari) are shown in Figure 2-2b, demonstrating that each internode is evenly shortened in dwarf13, relative to Shiokari. Therefore, the dwarf13 is dn-type rice mutant.

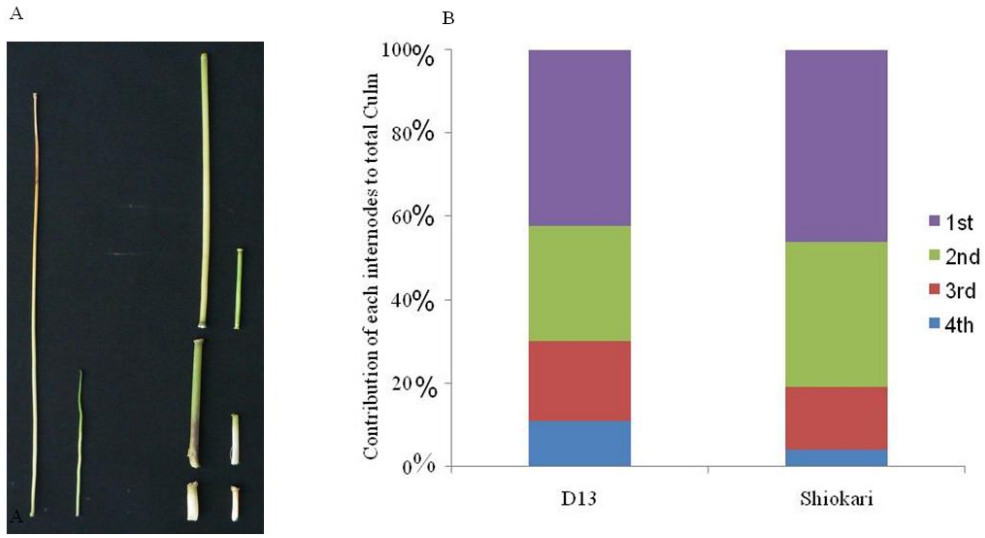
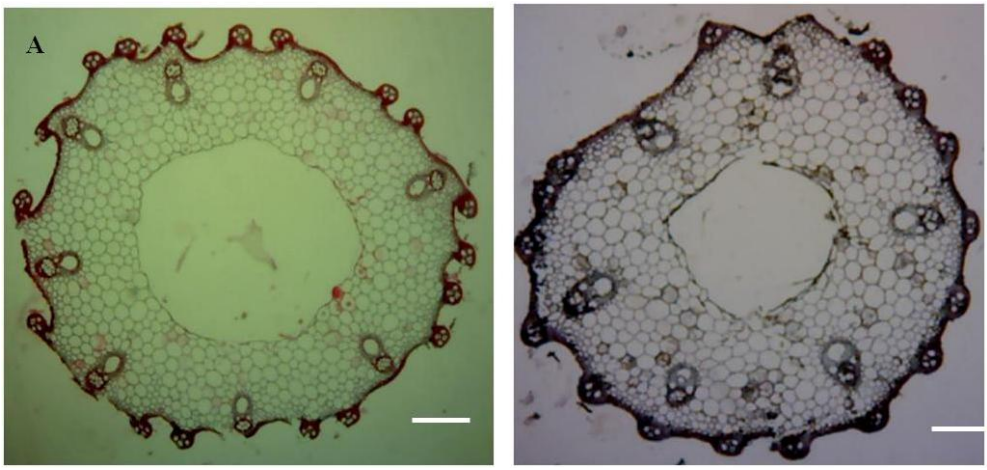
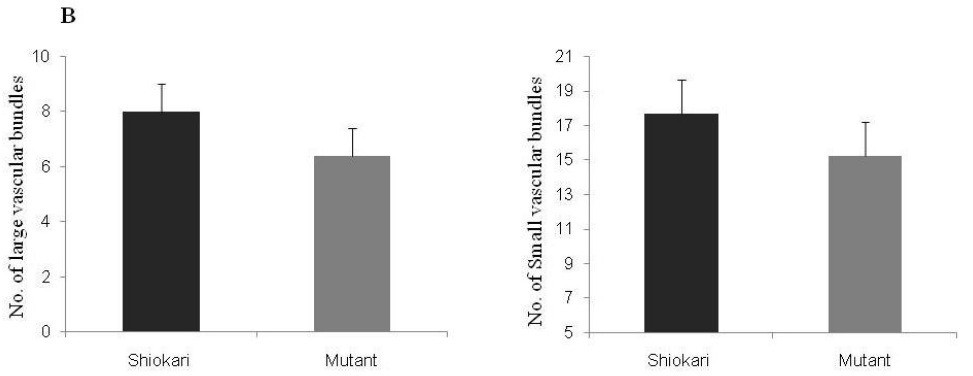


Figure 2-2. Internodes elongation pattern; **a** Schematic representation of upper four internodes of wild type (left) and dwarf13 (right), **b** Contribution of each internode to the culm length

Microscopic observations of the uppermost internodes

The short statures strengthen the mechanical support in the dwarf than wild type tall plants, as the vascular bundle system of the culmsprovides mechanical support for the body ofthe rice plant (Teale et al. 2006; Aohara et al. 2009). To investigatethe cell structure, we compared the peripheral vascular tissues and sclerenchyma cells of the uppermost internodes. Microscopic observation of cross-sections demonstrated that the number of both large and small vascular bundles were declined for mutant which resulted in the reduced diameterof the stem and pith cavity in the dwarf13 mutant (figs 2-3a, b). In longitudinal sections of the uppermost internodes, the observed cells were high in number and reduced size in the mutant than those wild type plants (figs. 2-3c). Taken together, these above observations suggest that the *d13* allele enhances meristematic activity and promotes cell proliferation leading to severe dwarfism in the mutant plants.


A


B
No. of large vascular bundles
10
8
6
4
2
0
Shiokari
Mutant
No. of Small vascular bundles
21
19
17
15
13
11
9
7
5
Shiokari
Mutant

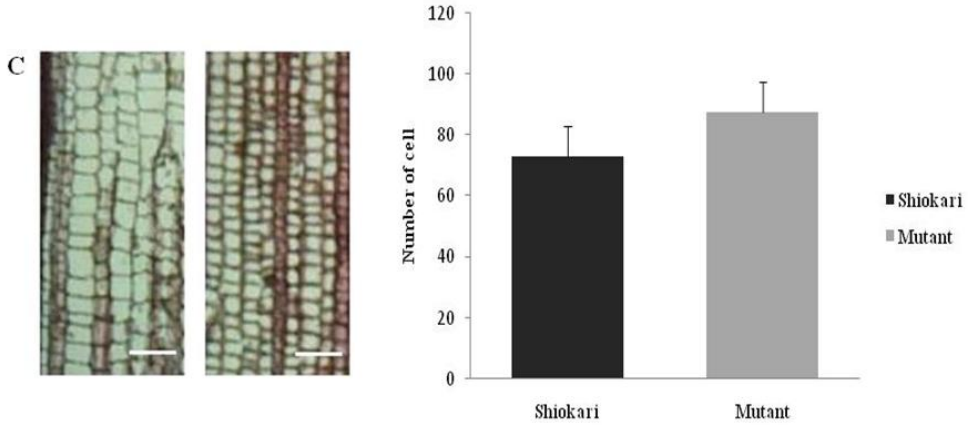

C
Number of cell
120
100
80
60
40
20
0
Shiokari
Mutant
Shiokari
Mutant

Figure 2-3. Microscopic observations of wild type (left) and dwarf mutant (right); **a** Cross-section, **b** Comparison of the number of large and small vascular bundles and **c** Longitudinal section of the upper most internodes and cell number, Bars 100 μm.

Evaluation of dwarf13 mutant in relation to GA_3 related physiological process

GA_3 plays a wide range of plant growth responses including seed germination, stem elongation, leaf expansion, induction of flowering, and pollen maturation (Richards et al. 2001; Thomas et al. 2005). One of the best known actions of GA_3 is the stimulation of shoot growth in rice (Matsukura et al. 1998). To determine the possible connection between the dwarfism in the dwarf13 mutant and the GA pathway, we observed the effects on the shoot elongation and the endosperm α-amylase activity induction by the GA_3 application to the mutant and wild type, both of which are GA related physiological processes (Mitsunaga et al. 1994).

The effects of GA application on the second leaf sheath elongation of the mutants were measured. The shoot elongation was started at 10^{-8} M and gradually increased the length with higher GA_3 concentrations. The elongation ratios were almost similar between dwarf13 and wildtype plants, and the response curve was also more or less similar with that of wild-type plants (fig. 2-4a).

To examine if any defect in the signal transduction of GA_3 exists in the dwarf13 mutant, agar plate assay for α-amylase activity was conducted using the embryoless half-seeds. The patterns of α-amylase activity were observed as white zones both in mutant and wild type only in the plate

containing GA_3 but the α-amylase activity in the mutant was lower than wild type (fig. 2-4b). The mutant showed a normal response to exogenous GA with respect to production of α-amylase. Therefore, the mutant belongs to the group E that is neither a GA deficient nor a GA sensitive according to Mitsunaga et al. (1994). This results indicated that the mutant was responsive to GA even at a reduced rate.

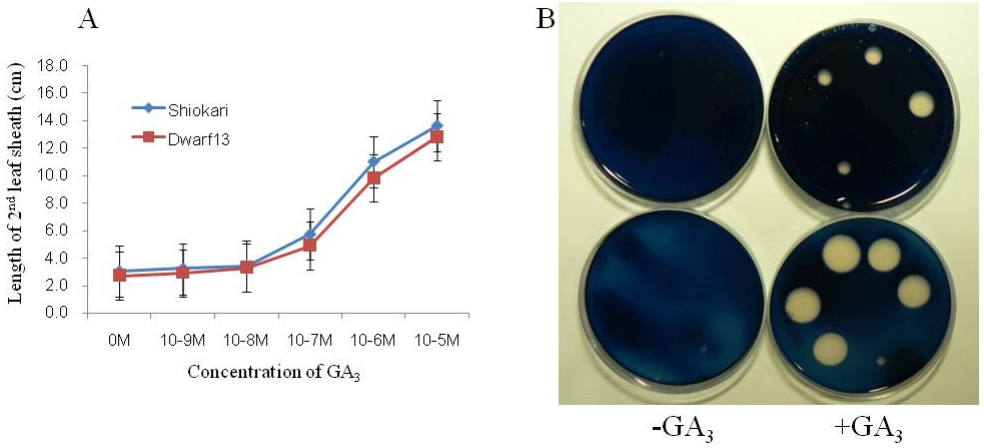


Figure 2-4. Relationship between dwarf13 mutant and GA_3; **a** Elongation of the 2nd leaf sheath in response to different GA_3 treatments. Error bars the SD from the mean (n=10) **b** the α-amylase production from embryoless half-seeds of Shiokari (bottom) and the Dwarf13 mutant (top)

To further examine the relationship between the *d13* gene and GA metabolic pathway, the expression of GA biosynthetic genes, including OsCPS1, OsKS1, OsKO1, OsKO2, OsKAO, OsGA20ox2, OsGA2ox3 and OsGA2ox1 were examined by using real-time qPCR. The results showed that the expression levels of OsCPS1, OsKS1, OsKO1, OsKAO, OsGA20ox2, OsGA2ox3 and OsGA2ox1 were significantly enhanced except OsKO2 in the dwar13 mutant. There might be another molecular reason behind the low expression level of OsKO2 in the mutant. The expression level of OsGA2ox1 which encodes GA 2-oxidase was also analyzed. GA2ox proteins function in the control of GA levels by inactivating the bioactive GAs. The results showed that the expression level of OsGA2ox1 increased in dwarf13 mutant. However, the mRNA levels of OsCPS1, OsKAO, OsGA20ox2, OsGA2ox3 and OsGA2ox1 in the mutant were 2-6 times higher than those in wild-type plants (Fig. 2-5). These results indicated that *d13* gene influenced the expression of GA biosynthetic genes in rice and might be involved in biosynthetic pathway.

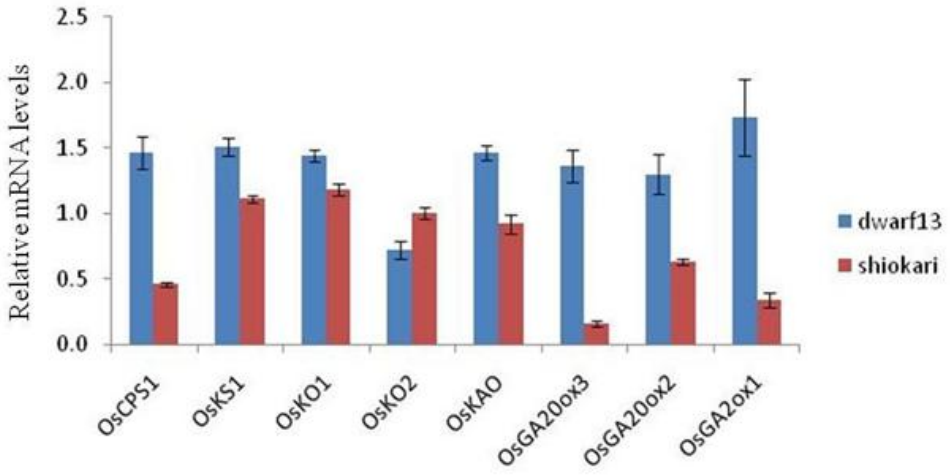


Figure 2-5. Expression analysis of the GA biosynthetic genes. The mRNA levels of OsCPS1, OsKS1, OsKO1, OsKO2, OsKAO, OsGA20ox2, OsGA2ox3 and OsGA2ox1 were measured by real-time qPCR

Genetic analysis of the dwarf13 mutant

To estimate whether the dwarf13 was controlled genetically by a single gene or multiple genes, two F_2 populations were developed from the crosses between the dwarf13 mutant and two wild-type varieties, Shiokari and Milyang23, respectively. All the F_1 plants from the two crosses displayed wild phenotype, and also their F_2 progenies have a segregation ratio of 3:1 between wild-type and mutant plants (Table 2-3). Therefore, the dwarfism of the dwarf13 mutant is controlled by a single recessive gene in rice.

Table 2-3. Genetic segregation analysis of the dwarf13 mutant in the two F_1 and F_2 populations

Cross combination	F_1 plants			F_2 plants			χ^2 (3:1)
	Total	WT	mutant	Total	WT	mutant	
d13/Shiokari	17	17	0	405	299	106	0.29
d13/Milyang23	21	21	0	1273	951	322	0.06

Isolation of the *d13* gene by positional cloning

We identified the molecular markers linked to the *d13* gene by bulked segregant analysis (Michelmore et al. 1991). There were 163 STS markers used which dispersed over 12 chromosomes. Among the 163 primers, it was found that there were 64 primers, which could trace the polymorphism between dwarf13 mutant and Milyang23. Furthermore, after BSA with the F_2 populations from the cross of dwarf13 and Milyang23, the primer, S0900A was found to be linked with the dwarf trait on the short arm of chromosome 9.

Based on the genotype and phenotype data, the *d13* locus was delimited in about 3.2 cM region between S0900A and S0903.2 markers by rough mapping (fig. 2-6a). To refine the position of the *d13* gene, 2 SSR and 13 additional STS markers were designed within the flanking region based on the sequences differences available at the rice genomic databases. We further narrowed the d13-containing region to a 265 kb interval by using 1273 F_2 individuals. Finally *d13* gene was flanked by the two STS markers DMR-3 and S5789 around the centromeric region of chromosome 9 (figs. 2-6b, c). We couldn't narrow down the flanking region more precisely because of less number of F_2 individuals and the centromeric region with low meiotic recombination (Cheng et al. 2001) and abundance long satellite repeats and retrotransposons (Zhu et al. 2007). Finally, based on the available sequence annotation databases (http://www.tigr.org and http://www.gramene.org), there are 30 predicted genes annotated in this region (fig. 2-6d).

To isolate the candidate of *d13* gene, the delimited DNA region is being sequenced and compared between dwarf13 and its wild type Shiokari.

Among the 30 candidate genes within the flanking region, eight genes with known function, ten are expressed protein, six hypothetical proteins, and six are retrotransposon protein. But it's hard to clone due to the abundance long satellite repeats and retrotransposons in the centromeric region (Zhu et al. 2007). Now, sequencing of the other candidate genes is ongoing to find the responsible gene for dwarfing mutant. Hopefully, molecular identification of responsible gene for small grain dwarf will provide the new clue for dwarfism and the relation GA in rice.

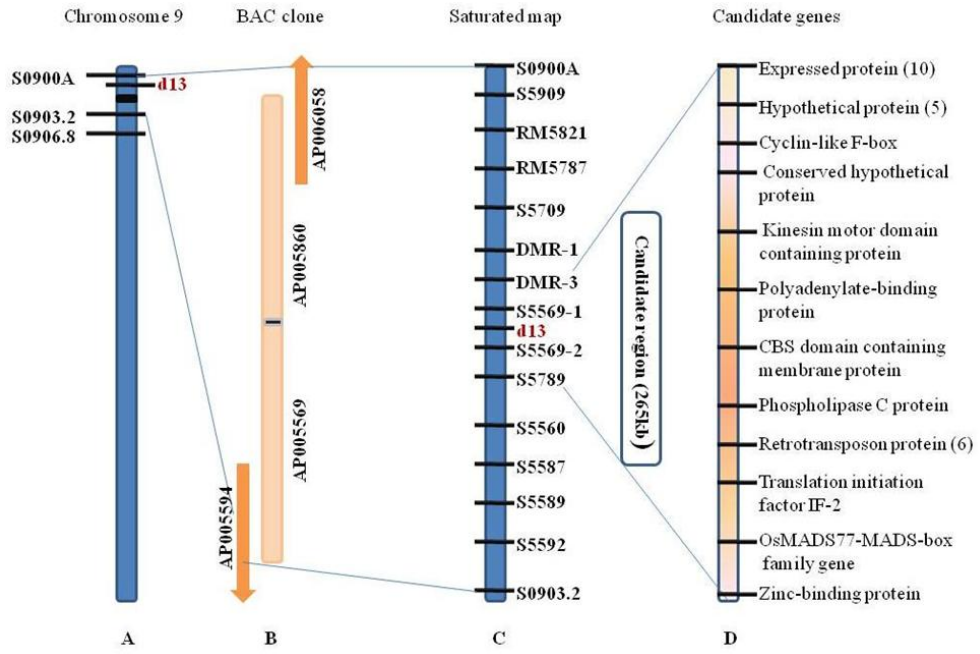


Figure 2-6. Genetic map of the region containing the *d13* locus; **a** Linkage map of d13 with flanking markers on chromosome 9 **b** The BAC contigs in the *d13* gene region **c** Fine mapping of the *d13* locus and **d** Candidate genes with in the 265 kb DNA region between two markers DMR-3 and S5789, respectively

DISCUSSION

Here, we demonstrated that *d13* is involved in the regulation of small grain dwarf phenotype through GA mediated physiological process. Analysis of the agronomic and morphological characteristics revealed that the *d13* mutations wield pleiotropic effects on the rice plant development, including plant height, smaller and erect panicle, short rachis-branches, reduced seed size, increased tiller number, short leaf blade with yellowish stripe, late and asynchronous heading, and stronger root system. These above observation suggested that the dwarf13 mutant involved in several morphological defects. In addition, each internode is evenly shortened via inhibiting the elongation of the uppermost four internodes and belongs to the dn-type dwarf mutant in rice based on the classification by Takeda (1977). In rice, the vascular bundle system of the culms provides mechanical support for the body ofthe rice plant (Aohara et al. 2009; Teale et al. 2006; Ye 2002). In this study, the uppermost internodes of the dwarf13 mutant had reduced number of vascular bundles resulted in small pith cavity and reduced cell size than the wild type indicated that the *d13* allele enhances meristematic activity and promotes cell proliferation leading to severe dwarfism in the mutant plants.

Gibberellins have an important role in the regulation of many physiological processes in the growth and development of plants. Most of the reported dwarf mutants were related to the biosynthesis or signaling pathways of gibberellins and few of them are brassinosteroid-related dwarf mutants (Kurata et al. 2005). In our GA related experiments, first, endosperm α-amylase activity induction by the GA_3 application in the dwarf13 mutant showed reduced α-amylase activity in endosperm but the exogenous application of GA_3 on the second leaf sheath showed strong response to GA

that more or less similar with that of wild-type. Based on the two GA mediated process, the second leaf sheath elongation and α-amylase activity induction assays showed that *d13* gene was related to the pathway of GA metabolism in rice. In our knowledge, recently some GA related dwarf genes were reported (Asano et al. 2009; Ashikari et al. 1999; Li et al. 2010; Liu et al. 2008; Qin et al. 2008; Ueguchi-Tanaka et al. 2000; Zou et al. 2005) but all genes are located on different chromosomes. Since the plant hormone GA has been shown of growth and developmental processes, including stem elongation, seed germination, and flowering (Yamaguchi 2008; Sakamoto et al. 2004; Grennan 2006; Ueguchi-Tanaka et al. 2005) a defect in its metabolism and/or signaling is most likely responsible for the dwarf phenotype. The GA biosynthetic genes, OsCPS1, OsKS1, OsKO1 and OsKAO, the expression of which was independent of the levels of GA, were also significantly increased in dwarf13 mutant. These observations indicated that *d13* gene affected the expression of the GA biosynthetic genes. Furthermore, the expression level of OsGA2ox1 was up-regulated in the dwarf13 mutant which functions in the control of GA levels by inactivating the bioactive GA (Sakamoto et al. 2004). Our results were consistent with earlier studies by Li et al. (2010). Taken together with all GA related data in this study, suggested that *d13* gene might be involved in unknown GA related dwarfing mechanism. But how the d13 gene is involved in biosynthetic pathway and dwarfing mechanism remains unknown and our further studies are underway to better understand their exact functions during rice growth and development.

Genetic analysis revealed that the dwarf phenotype was controlled by a single recessive mutation. By using several STS markers, the *d13* locus was fine mapped to 265 kb around centromeric region on the short arm of

chromosome 9. The centromeric region was proved with low meiotic recombination (Cheng et al. 2001) and hard to clone due to the abundance long satellite repeats and retrotransposons (Zhu et al. 2007). Several approaches have been used to identify genes located within the centromeric region. *Ghd7* gene based on bioinformatics approach and genetic complementation (Xue et al. 2008); large population used for *BRITTLE CULM1* (*BC1*) gene (Qian et al. 2001; Li et al. 2003). Based on linkage analysis, the *d13* gene was finally located in the 265 kb physical distance within the newly developed flanking markers namely, DMR-3 and S5569-1 having 30 predicted genes in this region.

Mapping analysis revealed that the only OsCPS3, GA related gene was mapped onto chromosome 9. Based on the location, we presumed that the *d13* gene is non-allelic with the OsCPS3 gene (Sakamoto et al. 2004). Our above discussion indicated that dwarf13 was a new small grain dwarf mutant probably involved in a different GA metabolic pathway. Further studies of the *d13* gene will be provided a new clue for dwarfism in rice.

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초록

벼에서단간및이삭끝퇴화돌연변이유전자의유전자지도작성및클로닝

엠디악터

벼는 세계에서 가장 중요한 작물이자 많은 아프리카, 아시아 사람들의 중요한 주식이다.전 세계 인구에 식량을 공급하기 위해서는작물의 생산과 생산성이 동시에증가되어야 한다.이상적인 초형을 가지고 있는 작물들은 수량 증가에 유리하기 때문에식물체 초형의 개선은 작물 육종에 있어서 매우 중요하다.벼에서 이삭의 구조와 식물체의 키는 초형의 중요한 요소들이고 이는 곧 수량을 결정한다.

이삭의 형태는 주로 지경과 영화(소수)의 배열에 의해 결정되고 직접적으로 수량에 여향을 미친다. 자포니카 재배종인 화청벼에 MNU 를 처리하여 이삭선단퇴화 (PAA-Hwa) 돌연변이를 유기하였다.이 돌연변이는 일반적인 생육환경하에서 식물체의 키가 약간 줄어들고,잎몸이 좁아지며 진한 녹색을 띠었다.또이삭 끝이 퇴화되며,이삭이 작아지고 곧추서는 등의 여러가지 비 정상적인 표현형을 보였다.유전분석 결과 이삭선단퇴화 현상은 단일열성 유전자에 의해 조절 되었고 이를 *paa-h* 로 명명하였다. *paa-h* 유전자는 4 번 염색체의 aptn3 과 S6685-1, 두 마커 사이에 위치하였고,이 사이에 존재하는 후보 유전자들의 염기서열을 분석한

결과 *Loc_Os04g56160* 유전자에서 단일염기변화가 일어났고 아미노산이 glycine 에서 glutamic acid 로 치환되었다. *Loc_Os04g56160* 유전자는 주로 이삭에서 발현되고, 951 개의 아미노산으로 이루어져있으며 Plasma membrane ATPase protein 을 암호화한다. *Loc_Os04g56160* 유전자의 T-DNA 라인은 이삭선단퇴화 표현형을 가지는 PAA-Hwa 돌연변이와 동일한 표현형을 보였다.

Dwarfism 은 작물육종에 중요한 농업형질 중에 하나이다. 왜냐하면 왜성 재배종들은 도복에 저항성이 있고 이것은 단자엽 식물, 특히 벼에서 수량 증가를 가져오기 때문이다.하지만 dwarfism 을 조절하는 유전적 메커니즘은 아직 잘 밝혀지지 않았고,대부분 돌연변이의 원인이 되는 유전자들 또한많이 알려지지 않았다. Shiokari 로부터 얻는 dwarf13 돌연변이의 특성을 연구하였는데,포장조건 하에서 돌연변이의 표현형은 정상인 것에 비해 식물체 키가 작아지고,분얼이 증가 했으며,잎 끝이 짧고 둥글어졌다.또잎몸에 노란색 줄무늬가 생겼고,출수기가 늦어지고 불균일해졌으며,근계가 잘 발달하였다.그리고짧고,바로 서는 이삭의 형태를 보였으며종자가 작아진 것을 관찰 할 수 있었다.이 돌연변이는 절간신장 패턴에 의해구분하는 Takeda 방법으로는 dn-type 에 속하고, GA 를매개하는 과정에 근거한,GA 반응으로 구분하는 Mitsunaga 방법에 의해서는 E group 에 속한다.OsCPS1, OsKS1, OsKO1, OsKAO, OsGA20ox2, OsGA2ox3 and OsGA2ox1 과 같은 GA 생합성과 관련된 유전자들의 발현은 dwarf13 돌연변이에서 증가하였다. 해부학적 관찰한 결과 dwarf13 돌연변이는 정상개체에 비해 유관속의 개수가 적었고,세포의 크기도 작았다.유전분석 결과 dwarf13 돌연변이는 단일열성 유전자에 의해 조절되었고,해당 유전자는 9 번 염색체의 동원체 부근의 DMR-3 과 S5789,두마커 사이(265kb)에 위치하는 것으로 나타났다.이 영역에는 30 개의 후보 유전자가 있는 것으로 예측되었다.

Paa-h 와 *d13* 유전자는 각각 이삭선단퇴화와 dwarfism 의 분자생물학적 기작의 연구를 위한 새로운 단서를 제공 할 것으로 기대한다.

핵심어: 벼, 이삭선단퇴화, Plasma membrane ATPase, dwarfism, 지베렐린, Map-based cloning

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