Running title: *Atlpk2β* in Axillary Shoot Branching via Auxin

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Arabidopsis Inositol Polyphosphate 6-/3-kinase (Atlpk2β) is involved in Axillary Shoot Branching via Auxin Signaling

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ABSTRACT

The Arabidopsis inositol polyphosphate 6-/3-kinase gene (*AtIpk2β*) is known to participate in inositol phosphate metabolism. However, little is known about its physiological functions in higher plants. Here, we report that *AtIpk2β* regulates Arabidopsis axillary shoot branching. By overexpressing *AtIpk2β* in wild-type and mutants, we found that overexpression of *AtIpk2β* leads to more axillary shoot branches. Further analysis of *AtIpk2β* overexpression lines showed that axillary meristem forms earlier and the bud outgrowth rate is also accelerated, resulting in more axillary shoot branches. *AtIpk2β* promoter/β-glucuronidase (GUS) fusions (*AtIpk2β::GUS*) expression pattern is similar to that of the auxin reporter *DR5::GUS*. Moreover, *AtIpk2β* can be induced in response to exogenous IAA treatments. In addition, *AtIpk2β* overexpression plants exhibit auxin-related phenotypes and are more resistance to exogenous auxin treatment. Further analysis employing RT-PCR shows that some genes involved in auxin biosynthesis (*CYP83B1*), auxin transport (*PIN4*) and auxin-mediated branching regulation (*MAX4* and *SPS*) are regulated by *AtIpk2β*. Taken together, our data provide insights into a role for *AtIpk2β* in axillary shoot branching through auxin signaling pathway.
INTRODUCTION

Inositol 1,4,5-trisphosphate 3-kinase (IP3K) plays an important role in maintaining Ca^{2+} homeostasis in animal cells by regulating the levels of inositol 1,4,5-trisphosphate (IP_3) and inositol 1,3,4,5-tetrakisphosphate (IP_4) (Berridge, 1993). Inositol phosphates (IP) are a group of mono- to polyphosphorylated inositols and they have important secondary messengers in eukaryotic cells. IP_3 and IP_4 are second messengers that regulate cytosolic calcium concentration in animal cells by different pathways (Mignery et al., 1992; Berridge, 1997; Hill et al., 1998). Inositol 1,2,3,4,5,6-hexakisphosphate (IP_6), also known as phytate, is associated with human neutrophil functions (Eggleton et al., 1991) and plant seed germination (Loewus and Murthy, 2000). Several cDNAs encoding IP3Ks have been isolated and characterized from rat (Choi et al., 1990; Thomas et al., 1994), human (Takazawa et al., 1991a, 1991b; Dewaste et al., 2000), chicken (Bertsch et al., 1999), nematode (Clandinin et al., 1998) and yeast (Odom et al., 2000). Mammalian IP3Ks were involved in diverse processes including brain development (Mailleux et al., 1991), embryogenesis (Frederick et al., 2005), memory and learning (Kim et al., 2004), membrane traffic and Ca^{2+} homoeostasis (Soriano et al., 1997), oxidative stress resistance (Monnier et al., 2002). In Arabidopsis, there are two IP3ks (Atlpk2α and Atlpk2β) (Stevenson-Paulik et al., 2002; Xia et al., 2003). Both of the Arabidopsis IP3Ks recognize IP_3 as a substrate and display dual-specificity inositol polyphosphate 6-/3-kinase activities (Stevenson-Paulik et al., 2002; Xia et al., 2003). Such dual-specific activities are also found in yeast, where it regulates arginine metabolism (Odom et al., 2000). Interestingly, Atlpk2β was found to be located predominantly in the nucleus and complemented a yeast ipk2/arg82 mutant lacking a functional ArgR-Mcm1 transcriptional complex (Xia et al., 2003). These data suggest a possible role for Atlpk2β as a transcriptional control mediator in higher plants. The molecular cloning, biochemical properties and biological
functions of IP3Ks from animal, yeast and plant were reviewed (Xia and Yang, 2005). Recently, \textit{AtIpk2α} was reported to regulate pollen and root development (Xu et al., 2005). Stevenson-Paulik et al. demonstrated a novel role for \textit{AtIpk2β} in phytate synthesis (Stevenson-Paulik et al., 2005).

The pattern of axillary shoot branching and the growth of axillary shoots determine to a large extent the growth and developmental status of plants. In the majority of flowering plants, primary shoot apical meristem (SAM) is activated during embryogenesis and followed by the formation of additional meristems (Steeves and Sussex, 1989). The primary plant axis is provided by the SAM, whereas the architecture of the shoot system is further determined by the activation of axillary meristems. Compared to SAM, axillary meristems may initiate and then develop into either a branch or a blocked axillary bud (Stafstrom and Sussex, 1992; Evans and Barton, 1997). Axillary shoot branching generally involves two developmental stages: the formation of axillary meristems and the outgrowth of axillary buds. In Arabidopsis, maize, tomato, petunia, and pea, various mutants with axillary shoot branching defects have recently been identified (Shimizu-Sato and Mori, 2001; Ward and Leyser, 2004; McSteen and Leyser, 2005). Although the precise mechanisms controlling axillary shoot branching are poorly understood, some factors are demonstrated to be important to this process.

Plant hormones, mainly auxin and cytokinins are important regulators of axillary shoot branching. Physiological studies indicate that IAA acts as repressors of axillary bud growth, whereas cytokinins promote it (Cline, 1997; Napoli et al., 1999; Chatfield et al., 2000). Some auxin biosynthetic genes including \textit{NIT1}, \textit{TRP}, \textit{YUCCA}, \textit{CYP83B1}, \textit{CYP79B2} and \textit{CYP79B3} have been identified (Normanly et al., 1993; Hillebrand et al., 1998; Bak et al., 2001; Zhao et al., 2001, 2002). \textit{CYP83B1} is a regulator of auxin production (Bartel et al., 2001; Woodward and Bartel, 2005). \textit{PIN} genes (\textit{PIN} 1, \textit{PIN} 2, \textit{PIN} 3, \textit{PIN} 4, \textit{PIN}
6 and PIN 7) mediate the amount and direction of polar auxin transport (Chen et al., 1998; Galweiler et al., 1998; Friml et al., 2002a, 2002b; Friml, 2003; Woodward and Bartel, 2005). PIN4 is essential for auxin gradient and is important for pattern formation in the root tip (Friml, 2003). Auxin synthesis or transport is required for axillary meristem formation and development. Mutations that disrupt auxin synthesis or transport, affect axillary meristem formation and development (Okada et al., 1991; Bennett et al., 1995; Przemeck et al., 1996; Reinhardt et al., 2003). Some mutants, like auxin resistant (axr1), more axillary branching (max4), supershoot (sps), exhibit phenotypic alteration in axillary shoot branching. The axr1 mutant promoted axillary shoot growth and not affect axillary meristem formation (Stirnberg et al., 1999). MAX4 is required for the production of a mobile branch-inhibiting signal. It is a not-yet-identified shoot multiplication signal (SMS) that interacts with auxin to inhibit branching (Sorefan et al., 2003; Bainbridge et al., 2005; Beveridge, 2006). The SPS acts as a modulator of cytokinin metabolism. The sps mutant affects axillary meristem initiation and bud outgrowth, resulting in more shoots (Tantikanjana et al., 2001).

Arabidopsis IP3K (AtIpk2α and AtIpk2β) expressed in stem, leaf, stigma, siliques, and fast-growing regions including root tips and root hairs (Xia et al., 2003; Xu et al., 2005), which implied that Arabidopsis IP3K may play important roles in plant growth and development. As expected, AtIpk2α transgenetic plants exhibited superiority in pollen germination, pollen tube growth, root growth and root hair development (Xu et al., 2005). However, the physiological functions of AtIpk2β in plant still remain unknown.

In this study, we demonstrate a novel role for AtIpk2β in axillary shoot branching. Moreover, we investigated the correlation between AtIpk2β expression pattern and in vivo auxin reporter DR5::GUS as well as the responses of AtIpk2β overexpression plants to IAA treatments. Finally, we analyzed the expression of auxin-related genes in AtIpk2β overexpression
plants. Our results suggest that Atlpk2β plays a role in axillary shoot branching through auxin signaling pathway.
RESULTS

Overexpression of *AtIpk2β* Generates More Axillary Shoot Branches

We have previously shown that the *AtIpk2β* gene (AGI locus no. At5g61760) is expressed in various Arabidopsis organs including roots, stems, leaves, and flowers (Xia et al., 2003). To further analyze the physiological functions of *AtIpk2β*, we overexpressed the *AtIpk2β* gene in Arabidopsis. Eleven *AtIpk2β* overexpression plants named OX-2, OX-3, OX-5, OX-8, OX-9, OX-15, OX-25, OX-26, OX-33, OX-35, OX-64 were identified and confirmed by Northern blot analysis (Fig. 1A). The expression levels of the *AtIpk2β* overexpression lines varied (Fig. 1A). Six of the *AtIpk2β* overexpression lines displayed elevated levels of *AtIpk2β* protein as determined by Western blot analysis using an antibody raised against MBP-*AtIpK2β* fusion protein (Fig. 1B). After two generations of segregation (seeds were germinated on MS medium containing hygromycin), homozygous *AtIpk2β* overexpression plants were selected for further studies. Two overexpression lines (OX-9 and OX-26) exhibiting a relatively high *AtIpk2β* protein level were chosen for the following experiments (Fig. 1B). Compared to wild-type, we found that branching of axillary shoots was more pronounced in the overexpression lines (Fig. 2A). At maturity, *AtIpk2β* overexpression plants had increased axillary branches.

To determine the effects of *AtIpk2β* on shoot architecture precisely, we examined the shoots from wild-type and *AtIpk2β* overexpression plants. The *AtIpk2β* overexpression plants did not alter leaf initiation rate and flowering time during vegetative growth (data not shown). However, *AtIpk2β* overexpression plants started to produce more secondary inflorescences once they flowered. As summarized in Table 1, *AtIpk2β* overexpression lines produced twice as many total branches than wild-type (37.9±2.5 versus 13.4±0.6). Axillary shoot formation from the axils of cauline leaves of the primary bolt was also stimulated in *AtIpk2β* overexpression plants. Secondary branching was obviously affected...
as well: overexpression lines produced approximately three times of the wild-type secondary shoots developed from the axils of leaves of first-order shoots (Table 1). To qualify higher order branching, the ratio of the total number of branches divided by the number of first-order branches was calculated (Stirnberg et al., 2002). Atlpk2β overexpression plants show no significantly difference from the wild-type in this ratio (0.7±0.1 versus 0.6±0.1, n=10). All these data suggested that the growth of higher order branching was greatly promoted by overexpressing Atlpk2β.

We also obtained two T-DNA insertion lines of Atlpk2β, ipk2β-1 and ipk2β-2 (Fig. 1C). Homozygous T-DNA insertion lines were identified by PCR using Atlpk2β-specific primers 3K-f and 3K-r (Xia et al., 2003) (Fig. 1C). No PCR product was found in both mutant lines, while a 900-bp fragment was produced from wild-type genomic DNA (Fig. 1D). RT-PCR analysis did not detect Atlpk2β expression in both lines, while the expression of Atlpk2α was not suppressed (Fig. 1E). Interestingly, the T-DNA mutant lines did not show significant branching differences compared to wild-type (Fig. 2B). However, when ipk2β-1 mutant was transformed with Atlpk2β (see Experimental procedures), the expression of Atlpk2β increased in transgenic mutants, whereas the expression of Atlpk2α was not changed (Fig. 1F). The transgenic mutants produced more branches at maturity (Fig. 2B), similar to the Atlpk2β overexpression lines. These results imply that overexpressing Atlpk2β increases branching.

**Atlpk2β Overexpression Lines Displayed Earlier Timing of Axillary Meristem Formation and Increased Rate of Bud Outgrowth**

Axillary shoot branching includes two developmental steps: the formation of axillary meristems and bud outgrowth. To investigate if Atlpk2β is preferentially involved in either stage or both, we analyzed the early stages of axillary shoot development in plants grown in short photoperiods. It has been demonstrated
that axillary shoot development at each leaf position can be classified into three stages: axillary cell divisions, appearance of the axillary meristem and formation of the first axillary leaf primordium (Stirnberg et al., 2002). In wild-type, the node positions for stage 1 ranged between 15 and 20 nodes (median 18), for stage 2 ranged between 20 and 25 nodes (median 23) and for stage 3 ranged between 25 and 30 nodes (median 28) from the apex (data not shown). However, in 
\textit{Atlpk2}\textsubscript{β} overexpression plants, the ranges of node positions at which stages 1, 2 and 3 were closer to the apex (median 13 for stage 1; median 19 for stage 2 and median 22 for stage 3). This result suggests that \textit{Atlpk2}\textsubscript{β} obviously affected the timing of axillary meristem formation and increased the rate of bud outgrowth subsequent to meristem initiation, which demonstrates that overexpression of \textit{Atlpk2}\textsubscript{β} affects the two stages of axillary shoot branching and results in more branching.

\textbf{\textit{Atlpk2}\textsubscript{β} Expression Pattern Are Similar to That of \textit{DR5::GUS}}

We have previously generated an \textit{Atlpk2}\textsubscript{β}::\textit{GUS} fusion reporter gene and it was shown that the \textit{Atlpk2}\textsubscript{β} gene is expressed throughout various Arabidopsis tissues (Xia et al., 2003). Particularly, GUS activity was detected in root tips, root hairs, root vasculature, vascular bundles of young leaves and emerging lateral root primordia with their associated vascular tissue (Fig. 3A). Close examination of 5-day-old seedlings revealed strong GUS staining at the distal end of emerging leaf primordia, young stipules, vasculature and the tips of the cotyledons, but not throughout emerging leaves (Fig. 3,B-D). The staining was also present in leaf axils and the basal part of axillary buds (Fig. 3E).

Furthermore, we observed GUS activity in root tip more carefully. GUS staining was observed in epidermis, endodermis, stele, quiescent center (QC), columella cells, with a stronger expression under the QC which followed the expression pattern of the auxin reporter \textit{DR5::GUS} (see Supplemental Fig. 1; Ulmasov et al.,
In Arabidopsis, axillary shoot branching is initiated at the shoot apex with the formation of axillary meristems (Hempel and Feldman, 1994). To understand the mechanisms of how Atlpk2β contribute to axillary meristem initiation and branch bud formation, we further investigated its spatial and temporal expression patterns in mature Arabidopsis plants. Initially, Atlpk2β::GUS expression was observed at the leaf axils, before any gross morphological changes became evident (Fig. 3F). Subsequently, strong GUS activity was maintained in leaf axils during the formation of axillary shoots (Fig. 3, G-I). GUS activity was detected in the protuberance and axillary meristems, and extended to the entire branch bud including the axillary leaf primordia and young leaves. Branching-related gene MONOCULM 1 (MOC1) has been reported to play an important role in controlling rice tillering by initiating axillary buds and promoting their outgrowth (Li et al., 2003). Our observation of GUS staining was similar to the expression pattern of MOC1 gene during rice tillering. In consistent with accelerated branching in Atlpk2β overexpression lines, these results suggest that Atlpk2β play an important role in the initiation of axillary meristems and bud outgrowth, probably through auxin signaling pathway.

**Atlpk2β is an Auxin Inducible Gene and is Important for Auxin Signaling Pathway**

The coincidence of Atlpk2β::GUS expression with DR::GUS distribution implies the connections of Atlpk2β and auxin signaling pathway. To test this hypothesis, the effects of exogenous IAA on Atlpk2β expression were analyzed using Atlpk2β::GUS transgenic seedlings. The expression of Atlpk2β was enhanced in roots after treatment with 1 µM IAA (Fig. 4A). Expression of Atlpk2β became stronger when the concentration of exogenous IAA was increased from 1 µM IAA to 10 µM IAA (Fig. 4A). We also examined Atlpk2β expression when
treated with 40 µM IAA. Figure 6A shows that *Atlpk2β* expression increased after 0.5 h treatment, whereas the expression of *Atlpk2α* remained virtually unaffected (Fig. 6A). These results indicate that *Atlpk2β* is an IAA-inducible gene while *Atlpk2α* is not.

To test whether *Atlpk2β* is necessary for normal endogenous auxin signals, we analyzed many developmental aspects that are thought to correlate with auxin signals. Consistent with the hypothesis, the petioles of cotyledons were more elongated in light-grown *Atlpk2β* overexpression plants (Fig. 4B). In addition, when grown in dark for 6 days, the hypocotyls of *Atlpk2β* overexpression seedlings were significantly longer than those of wild-type (OX-9: 11.9 mm; OX-26: 12.2 mm; wild-type: 8.9 mm), although the differences in hypocotyl lengths were less evident in light-grown seedlings (Fig. 4C). These results suggest that *Atlpk2β* overexpression lines altered auxin-related processes, indicating that *Atlpk2β* is involved in auxin signaling pathway.

To confirm our hypothesis, we further analyzed the phenotypes of light- and dark-grown seedlings in response to exogenous IAA treatment (Fig. 5). Exogenous IAA has been shown to inhibit the elongation of the primary root (Evans, 1984) and to stimulate lateral root formation (Katsumi et al., 1969). Figure 5A illustrates the effects of IAA application on primary root elongation in wild-type and *Atlpk2β* overexpression plants. The root lengths of *Atlpk2β* overexpression seedlings were longer than that of wild-type at different IAA concentrations. As IAA concentrations increase, root length is reduced in both wild-type and *Atlpk2β* overexpression plants (Fig. 5A). Compared with wild-type, whose roots were evidently inhibited by exogenous auxin, the *Atlpk2β* overexpression plants exhibited much less inhibition (Fig. 5A). At an IAA concentration of 0.1 uM, the root length of wild-type seedlings was ~71.4% of that of untreated seedlings, whereas *Atlpk2β* overexpression roots displayed much less inhibition, with root length being ~94.7% (OX-9) and 90.5% (OX-26).
of that of untreated controls. When auxin was supplemented at a concentration of 10uM IAA, wild-type roots were severely inhibited to ~25% of the length of untreated seedlings. By contrast, Atlpk2β overexpression roots showed less inhibition, with root length being ~36.8% (OX-9) and ~28.6% (OX-26) of that of untreated controls respectively (Fig. 5A). We also examined the lateral root density of wild-type and Atlpk2β overexpression seedlings at different concentrations of IAA (Fig. 5B). Atlpk2β overexpression plants show more lateral roots and higher relative lateral density than wild-type (Fig. 5B). However, on media containing increasing concentrations of IAA, the relative lateral density became lower in Atlpk2β overexpression plants than in wild-type (Fig. 5B). All of these results suggest that Atlpk2β inhibits IAA responses in roots to some extents. We also analyzed the outgrowth of cauline lateral inflorescences from excised nodes as described by Chatfield et al (2000), and found that Atlpk2β overexpression lines also altered exogenous IAA inhibition of excised lateral inflorescences outgrowth (data not shown).

**Atlpk2β Regulates the Expression of Auxin-Related Genes**

The expression level of some auxin-related genes was also detected in Atlpk2β overexpression line OX-26 and wild-type. RT-PCR experiments showed that the expression of auxin-biosynthesis genes NIT1, TRP, YUCCA, and CYP79B2 was not changed, while the expression of CYP83B1 is decreased in Atlpk2β overexpression plants (Fig. 6B). In addition, the expression of some auxin-transport genes was also detected. Compared to the unchanged expression of PIN1, PIN2, PIN3, and PIN7, the expression of PIN4 is much stimulated in Atlpk2β overexpression plants (Fig. 6B). The change of CYP83B1 and PIN4 demonstrated that auxin biosynthesis and transport were modified in Atlpk2β overexpression plants, consistent with their altered IAA responses (Fig. 5).
Finally, we compared the expression of other auxin-related genes. Figure 6C showed that the expression of MAX4 and SPS was decreased in overexpression plants, while the expression of AXR1 was not changed. MAX4 and SPS are required for auxin-mediated bud inhibition and outgrowth (Tantikanjana et al., 2001; Bainbridge et al., 2005). The decreased expression of MAX4 and SPS indicates that bud initiation and outgrowth are improved, which is consistent with increased branching phenotype in Atlpk2β overexpression lines. Furthermore, decreased expression of MAX4 suggests that Atlpk2β functions upstream of MAX. The AXR1 is one of the auxin-response genes (Stirnberg et al., 1999). The AXR1-mediated auxin signaling plays important roles in auxin signal transduction. To illustrate if Atlpk2β was involved in AXR1-mediated auxin signaling, we analyzed the expression level of Atlpk2β in axr1-3 mutant. Figure 6D shows that there is no significant difference between axr1-3 and wild-type (Fig. 6D). Furthermore, Figure 6C shows that the transcriptional level of AXR1 was also not changed in Atlpk2β overexpression line (Fig 6C). These results indicate that there is no correlation between Atlpk2β and AXR1 in transcriptional level.
DISCUSSION

We previously reported the isolation and identification of Arabidopsis \textit{Atpk2}\textbeta, a homolog of animal \textit{IP3K} genes (Xia et al., 2003). However, little is known about its physiological functions in higher plants. The data presented here provide evidence for a role of \textit{Atpk2}\textbeta in axillary shoot branching through auxin signaling pathway.

Auxin–related mutants fall into three classes on the basis of whether they affect meristem initiation, outgrowth (e.g. \textit{more axillary growth [max]}) or both (e.g. \textit{supershoot/bushy [sps]}, \textit{monoculm1 [moc1]}) (Tantikanjana et al., 2001; Li et al., 2003; Ward and Leyser, 2004). Our results demonstrate that \textit{Atpk2}\textbeta affect both axillary meristem initiation and outgrowth. Overexpressing \textit{Atpk2}\textbeta in wild-type and mutant plants yields more axillary shoot branches (Fig. 2). We found that more shoot buds are generated at axils of cauline leaves and rosette leaves in \textit{Atpk2}\textbeta overexpression lines (Table 1). In addition, higher order branches from nodes on the inflorescence are also promoted, while the wild-type lacks axillary meristem at these higher order nodes (data not shown). The ranges of node positions at which axillary cell divisions and appearance of the axillary meristem were seen closer to the apex in \textit{Atpk2}\textbeta overexpression lines than in wild-type. These observations indicate that enhanced axillary shoot formation in \textit{Atpk2}\textbeta overexpression plants is determined to a large extent by earlier formation of axillary meristems. This conclusion is further supported by \textit{Atpk2}\textbeta::\textit{GUS} studies (Fig. 3). High levels of GUS activity are detected in axils of leaf primordia before any morphological alteration and during axillary bud formation. This expression pattern is similar to that of \textit{SPS}, which negatively regulates axillary meristem formation and growth (Tantikanjana et al., 2001). RT-PCR analysis showed that the expression of \textit{SPS} was decreased in \textit{Atpk2}\textbeta overexpression plants. These results support the involvement of \textit{Atpk2}\textbeta in axillary meristem initiation. The rate of bud outgrowth was increased in \textit{Atpk2}\textbeta overexpression plants compare to
wild-type. GUS staining was also observed after axillary meristem initiation (Fig. 3, H-I). High GUS activity was also detected in entire axillary buds during their growth and extended to young leaves. This spatial and temporal expression patterns is similar to that of the *MONOCULM 1 (MOC1)* gene in rice (Li, et al., 2003). *MOC1* is an important controller of rice tillering and its mutant shows defects in the formation of tiller buds and bud outgrowth (Li et al., 2003). Another critical gene involved in axillary outgrowth is *MAX4*, and *max4* mutants exhibit more branching (Sorefan et al., 2003). Interestingly, we observed a decrease in *MAX4* transcription level in *AtIpk2β* overexpression plants. Although the detailed pathways are not known, our data suggests that *MAX4* is regulated by *AtIpk2β* during bud outgrowth. Further analysis of branching shows that the proportion of vegetative nodes that are potential sites to produce a new branch was higher in *AtIpk2β* overexpression plants than in wild-type (Table 1). All of these data demonstrate that *AtIpk2β* also functions in axillary bud outgrowth.

Apical dominance is another broadly known mechanism that controls axillary bud growth (Thimann and Skoog, 1934; Thimann, 1937). In Arabidopsis, the growth of axillary buds is inhibited by auxin derived from the apical bud. However, auxin does not enter lateral buds and a second signal is involved in the repression of lateral shoot outgrowth. Auxin distribution analysis shows that the distal end of leaf primordia and stipules of 4-day-old seedlings have high levels of IAA (Avsian-Kretchmer et al., 2002). IAA also accumulates in the leaf tip. GUS activity presents the coincidence between the expression patterns of *AtIpk2β* and IAA distribution. Moreover, *AtIpk2β* gene expresses throughout development with the highest level in root, inflorescences, and flowers, suggesting that *AtIpk2β* has a broad role in many auxin-regulated processes (Xia et al., 2003). At present, although we do not know how *AtIpk2β* affects these processes, several observations raised the possibility that *AtIpk2β* participates in auxin signaling pathway. First, *AtIpk2β* can be induced by exogenous IAA (Fig.
4A and 6A). Second, *AtIpk2β* overexpression lines display altered IAA responses. Root elongation, lateral root formation and excised lateral inflorescence outgrowth under exogenous IAA are affected in *AtIpk2β* overexpression plants (Fig. 5). Third, *AtIpk2β* overexpression lines display decreased expression of *CYP83B1* and enhanced expression of *PIN4*. *CYP83B1* decrease the level of IAA by distributing indole-3-acetaldoxime to the glucosinolate pathway. It has been shown that *CYP83B1*-deficient plants overaccumulated IAA, indole-3-acetaldehyde, and IAA-Asp, whereas *CYP83B1* overexpression resulted in high indole glucosinolates and low IAA (Bartel et al., 2001). *PIN4*-dependent auxin transport actively maintains the auxin gradient in roots, stabilizing it through a feedback loop (Friml, 2003). The *pin4* mutant affects endogenous auxin gradients and root pattern. These results indicate that overexpression *AtIpk2β* affects auxin distribution and accumulation. Furthermore, the expression level of *MAX4* and *SPS* was decreased in overexpression lines (Fig. 6). These two genes are required for auxin-mediated shoot branching (Tantikanjana et al., 2001; Sorefan et al., 2003; Bainbridge et al., 2005). The decreased expression of *MAX4* and *SPS* indicates an important role for *AtIpk2β* in axillary shoot branching by negatively regulating *MAX4* and *SPS*, although it is also possible that *AtIpk2β* additionally affects other regulators of axillary shoot branching. Our data indicate that *AtIpk2β* is an early responsive gene in regulating branching by auxin signaling pathway.

Although *AtIpk2β* is involved in auxin signaling pathway, the exact functions in branching systems are not clear. Classical branching hypothesis states that auxin regulate shoot branching in conjunction with secondary messengers, and the candidates for this signal include cytokinin and SMS (Sachs and Thimann, 1967; Li et al., 1995; Foo et al., 2005; Beveridge, 2006). The *SPS* gene acts as a modulator of cytokinin metabolism and *MAX4* is required for the production of SMS (Tantikanjana et al., 2001; Beveridge, 2006). The decreased expression of
MAX4 and SPS in AtIpk2β overexpression plants suggested that AtIpk2β is involved in the negative regulation of these two signals in branching systems. Auxin and the MAX-dependent hormone interact to inhibit branching. However, the MAX-dependent hormone is a novel regulator of auxin transport which regulate bud outgrowth independent of AXR1-mediated auxin signaling (Bennett et al., 2006). AtIpk2β is involved in auxin signaling and affects the expression of CYP83B1 and PIN4, which is involved in auxin-biosynthesis and auxin-transport respectively. Therefore, AtIpk2β should be also independent of AXR1-mediated auxin signaling. RT-PCR showed that the expression of AXR1 was indeed not changed in AtIpk2β overexpression plants. Moreover, we also detected unchanged expression of AtIpk2β in axr1-3 mutant (Fig. 6). These data indicates that AtIpk2β may be independent of AXR1-mediated auxin signaling pathway, at least in transcriptional level.

Two IP3K isoforms (AtIpk2α and AtIpk2β) exist in Arabidopsis. They exhibit high homology in sequences (84% similarity and 73% identity). Both of them have inositol polyphosphate 6-/3-kinase activities on IP3 and involved in regulating Arginine metabolism in yeast (Stevenson-Paulik et al., 2002; Xia et al., 2003). They also show similar expression patterns in seedling, leaf, root, and floral organs (Xia et al., 2003; Xu et al., 2005). In addition, ipk2β mutants and ipk2α antisense lines display no significant branching differences compared to wild-type (unpublished data). All these evidences suggest that AtIpk2α and AtIpk2β have potential redundant (or partially redundant) genetic function. This hypothesis is supported by DR5::GUS studies. We analyzed DR5::GUS activity in ipk2β mutants and mutant seedlings showed the similar GUS expression pattern to wild-type (see Supplemental Fig. 2). York’s group (Stevenson-Paulik et al., 2005) previously pointed out the redundancy between AtIpk2α and AtIpk2β. AtIpk2α was able to fully compensate the phytate production in ipk2β mutant tissues (Stevenson-Paulik et al., 2005). However, AtIpk2α and AtIpk2β
display differential roles in expression patterns in pollen tubes (Xu et al., 2005) and in phytate synthesis (Stevenson-Paulik et al., 2005). Our experiment shows the different expression between \textit{AtIpk2}\textsubscript{α} and \textit{AtIpk2}\textsubscript{β}. \textit{AtIpk2}\textsubscript{β} was induced by IAA, but \textit{AtIpk2}\textsubscript{α} was not (Fig. 6A). We also detected the different responses between the two isoforms under other hormone and stress treatments (unpublished data). Thus, \textit{AtIpk2}\textsubscript{α} and \textit{AtIpk2}\textsubscript{β} have not only overlapping but also unique functions.

In this report, we studied the physiological functions of \textit{AtIpk2}\textsubscript{β} in axillary shoot branching. It is known that auxin may influence shoot branching via multiple pathways (Bennett et al., 2006). It seems likely that \textit{AtIpk2}\textsubscript{β} regulates axillary shoot branching in \textit{Arabidopsis}, at least in part, by integrating auxin signaling pathway. More detailed analysis will lead to further understanding of the molecular mechanism of signaling pathway integration.
MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype C24 and Columbia were used in this study. SALK_025091 (ipk2β-1) and SALK_104995 (ipk2β-2) mutant lines were obtained from the Arabidopsis Biological Resource Center (ABRC) (Alonso, et al., 2003). Vector constructs and the production of Atlpk2β transgenic plants are described below.

Seeds were surface-sterilized with 70% ethanol for 5 min and then washing for 3 times with sterile water, and plated on solidified MS medium (Murashige and Skoog, 1962). After 3 days cold treatment at 4°C, seeds were germinated at 22°C with a 16h-light/8h-dark cycle at 60% humidity.

Vector Constructions and Arabidopsis Transformation

To create an Atlpk2β (gene locus no. At5g61760) overexpression construct, the Atlpk2β cDNA was cut from plasmid pmIP3K with XbaI-XhoI (Xia et al., 2003) and inserted via XbaI-SalI sites into pBinAR-HPT vector behind the CaMV 35S promoter. The resulting plasmid (pBin-IP3K) was first introduced into Agrobacterium tumefaciens by electroporation and then transformed Arabidopsis thaliana ecotype C24 by vacuum infiltration (modified from Grant et al., 1995). Transgenic plants were selected on MS medium containing 20 mg/L hygromycin (Calbiochem, CA, USA).

RNA Extraction and Northern-blot Analysis

Total RNA from leaves of 2-week-old overexpression plants and wild-type was prepared according to the protocol of Logemann et al. (1987). RNA (35ug) was separated electrophoretically on denaturing 15% (v/v) formaldehyde and 1.5% (w/v) agarose gels. Northern hybridizations were performed as described by Xia et al. (2003).
Protein Extraction and Western-blot Analysis

Leaves of 2-week-old plants were ground in liquid nitrogen and suspended in extraction buffer containing 0.1 m Tris-HCl (pH 8.0), 18% (v/v) glycerol, 10 mm MgCl₂, 14 mm β-mercaptoethanol, 1 mg/mL pepstatin, 1 mg/mL leupeptin, and 1 mg/mL aprotinin. After a brief vortexing, the suspension was centrifuged at 14,000rpm for 10 min and the supernatant was collected. Proteins were separated on 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane. Western blot was carried out essentially as described by Xia et al.(2003) using an antibody against MBP-AtIpK2β fusion protein at a 1:1,000 dilution.

Identification of T-DNA Insertion Mutants and Arabidopsis Transformation

Seeds of ipk2β-1 and ipk2β-2 were first screened on the MS plates containing 50 µg/mL kanamycin (Duchefa, Biochemie, the Netherlands). The homozygous T-DNA insertion mutants were identified by a PCR genotyping assay with the specific primers 3K-f and 3K-r (Xia et al., 2003) for each individual plant. RT-PCR was used to analyze the expression of AtIpk2β and the actin gene served as a control in mutant and wild-type.

To overexpress AtIpk2β in ipk2β-1 mutant, the coding region of AtIpk2β was amplified by PCR using PfTurbo DNA polymerase (Stratagene) with primers IP3K-f (5'-CCGCTCGAGAAGATGCTCAAGGTCCCTGAACACCAAG-3') and IP3K-r (5'-ATGGGCCC CGCCCGCTTCTCAAGTAGGAAGTAGATCG-3') from wild-type Columbia genomic DNA. The primers added XhoI (5' end) and Apal (3' end) restriction sites to the amplified fragment. The PCR product was inserted via XhoI-Apal sites into vector pGreenLC201, containing a 35S promoter followed by a 6×haemagglutinin tag. The resulting plasmid pGLC201-AtIpK2β was first introduced into Agrobacterium tumefaciens by electroporation and then was transferred to Arabidopsis thaliana ipk2β-1 mutant by vacuum infiltration.
Transgenic plants were selected using Basta solution (Invitrogen).

**Root Length and Lateral Root Density Measurement**

Seeds (n>12) were germinated vertically in light for 11 days and in dark for 5 or 6 days, respectively, and hypocotyl length of seedlings was measured. As for root length measurement, 4-day-old seedlings (n>12) were transferred from unsupplemented plates to vertical plates containing 0, 0.1, 1, 10µM IAA, and primary root length was measured and calculated after 3 days of growth. Experiments were repeated three times. As for lateral root density assay, 4-day-old seedlings (n>12) were transferred from IAA-free plates to vertical plates containing different concentrations of IAA (0, 0.01, 0.1, 1 µM). After additional 3 days, the number of emerged lateral roots was counted and divided by the length of the primary root and then compare with the seedlings grown on unsupplemented medium.

**GUS Staining Assay**

The *AtIpk2β::GUS* transgenic plants were described by Xia et al. (2003). Samples from 5-day-old seedlings treated with 0, 1, 10, 100 µM IAA were fixed under vacuum for 10 min in 50 mM sodium phosphate buffer, pH 7.0, 0.005% (v/v) Tween 80, and 0.3% (v/v) formaldehyde. Then, tissues were washed three times in 50 mM sodium phosphate and stained for GUS activity in 50 mM sodium phosphate, 10 mM EDTA, 0.01% (v/v) Tween 80, and 0.5 mg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronide. After staining overnight, the samples were washed three times with 50 mM sodium phosphate and fixed for 30 min at room temperature in 20% (v/v) ethanol, 5% (v/v) acetic acid, and 5% (v/v) formaldehyde. Chlorophyll containing tissues were cleared in a series of ethanol: water mixtures up to 80% (v/v) ethanol, in which samples were stored. Microscopic analysis was performed using an Olympus SZX-ILLB200.
microscope (Tokyo, Japan) with bright-field optics. All images were obtained with the same modifications and intensity parameters. Images were photographed using a CCD camera and processed using Adobe Photoshop (Adobe Systems, CA, USA).

**Reverse Transcript PCR (RT-PCR) Analysis**

Total RNA was extracted from shoot tissue by using TRIzol isolation reagent (Invitrogen, CA, USA) according to the manufacturer’s protocol. First-strand cDNA synthesis reactions were performed in a 25 µl reaction with 0.5 mM dNTPs (Takara, Dalian, China), 5 µl 5 × MMLV buffer, 20 unit of RNAsin (Promega, WI, USA), 2 µg of total RNA, 0.2 µg of oligo (dT)14 primer (Sangon, Shanghai, China) and 200 units of MMLV sense transcriptase (Promega), then diluted to 25 µl. Primer sequences: 3K-f and 3K-r for AtIpk2β (Xia et al., 2003); IPK-RT1 and IPK-RT2 for AtIpk2α (gene locus no. At5g07370) (Xu et al., 2005); AtActin-F (5’-CATCAGGAGGACTTGTACGG-3’) and AtActin-R (5’-GATGGACCTGACTCGTACACA-3’) for Actin (At2g37620); YUCCAF (5’-ACACGGTCCCCATCATCATTGTC-3’) and YUCCAR (5’-AAGCCAAGTAGGCACGGTATC-3’) for YUCCA (At4g32540); 79B2F (5’-CCGGTTTCGGTACGATTGTC-3’) and 79B2R (5’-TGCTTGGACCGCATCGTACACA-3’) for CYP79B2 (At1g05090); 83B1F (5’-AGGGAAAATGCATGGTGTC-3’) and 83B1R (5’-TTGGCCTGACATCGTACACA-3’) for CYP83B1 (At4g31500); NIT1F (5’-TTCGGTTTAGCGGTTGTC-3’) and NIT1R (5’-TGCTTGGACCGCATCGTACACA-3’) for NIT1 (At3g44310); Trp-2F (5’-TCGGTTTCGGTACGATTGTC-3’) and Trp-2R (5’-TTCGGTTTAGCGGTTGTC-3’) for Trp-2 (At5g54810); PIN1F (5’-AACAGGCGCATTGTCACCCG-3’) and PIN1R (5’-AACAGGCGCATTGTCACCCG-3’) for PIN1 (At1g73590); PIN2F
(5'-ATCAGGAAGGATCTCTATGG-3') and PIN2R
(5'-AATAGCTGCATTGTCACCCG-3') for PIN2 (At5g57090); PIN3F
(5'-TTACTGCCTGTGCTATAGT-3') and PIN3R
(5'-GAGTTACCCGAACCTAATCA-3') for PIN3 (At1g70940); PIN4F
(5'-TCATTGCTTTGTGGGAGAATCT-3') and PIN4R
(5'-ACCACTTAACTAGAAACTTCA-3') for PIN4 (At2g01420); PIN7F
(5'-CGGTAAACATAATGCCACCA-3') and PIN7R
(5'-TCTAGTTGCCTCCACTAATC-3') for PIN7 (At1g23080); AXR1F
(5'-CGTTGATTACATAACCCAT-3') and AXR1R
(5'-GGATTATTCAGGCGGAGG-3') for AXR1 (At1g05180); SPSF
(5'-GGTGCTAAGGCTGCTGTT-3') and SPSR
(5'-AAGGGTGGTATCTTGACG-3') for SPS (At1g16410); MAX4F
(5'-CTTCGGTGTAACCAGACC-3') and MAX4R
(5'-GCCTCGGATTCAAGGAGA-3') for MAX4 (At4g32810). Actin expression level was used as a quantitative control. All oligonucleotides used in this study were synthesized by Sangon Technologies Inc. (Shanghai, China). Usually 32 cycles were used for PCR reactions. The RT-PCR assay was done at least three times for each sample.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes. Obtaining any permissions will be the responsibility of the requestor.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AJ404678 (Atlpk2β).
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FIGURE LEGENDS

Figure 1. Molecular identification of \emph{AtIpk2β} overexpression plants and T-DNA insertion mutants.

A, Northern blot analysis. Total RNA was isolated from 2-week-old seedlings (35 µg/lane). Lane 1, wild-type.

   Lanes 2 to 8, different \emph{AtIpk2β} overexpression lines, showing an increased transcription level of \emph{AtIpk2β}.

B, Western blot analysis. Total protein was isolated from 2-week-old seedlings (25 µg /lane). Lanes 1 and 8: wild-type. Lanes 2 to 7: different \emph{AtIpk2β} overexpression lines, showing increased \emph{AtIpk2β} protein level.

C, Schematic gene structure of \emph{AtIpk2β} and the representation of T-DNA insertion sites.

D, Identification of homozygous mutant lines by PCR. There is no PCR product in homozygous mutants \emph{(ipk2β-1} and \emph{ipk2β-2)}, while there is a DNA band with predicted size of 900 bp in wild-type.

E, RT-PCR shows the abolished \emph{AtIpk2β} transcript expression in mutant lines, while the expression of \emph{AtIpk2α} was not altered.

F, RT-PCR experiment shows that transgenic plants \emph{(ipk2β-1/AtIpk2β)} express a stronger \emph{AtIpk2β} transcript level than wild-type.

Figure 2. Phenotype analysis of \emph{AtIpk2β} transgenic plants. Bar, 2 cm.

A, \emph{AtIpk2β} overexpression plants (OX-26, ten-week-old) show more axillary branches.

B, Adult (nine-week-old) transgenic plants \emph{(ipk2β-1/AtIpk2β)} containing the overexpression construct \emph{(pGLC201-AtIpK2β)} show more axillary branches than \emph{ipk2β-1} mutant as well as wild-type.

Figure 3. Histochemical analysis of \emph{AtIpk2β::GUS} expression.

A and B, GUS expression was observed in the hypocotyl/root junction, lateral root, root hairs, vasculature of the cotyledons and the tip of the cotyledons (arrow).

C and D, The distal end (arrow) of emerging leaf primordia displays strong GUS staining.

E, GUS expression is also observed in the basal part of axillary buds (arrowhead).

F to I, GUS staining in leaf axils at different stages during axillary shoot branching.

   GUS staining was performed in five-day-old (A, B, C, D) and six-week-old (E to I) plants.
**Figure 4.** *Atlpk2β* is induced by IAA and *Atlpk2β* overexpression lines show auxin-related phenotypes.

A, GUS staining shows that expression of *Atlpk2β* was enhanced in roots after treatment with IAA. 5-day-old seedlings were exposed to different concentrations of IAA for 3 h before staining.

B, The petioles of cotyledons (one-week-old) are more elongated in *Atlpk2β* overexpression line (OX-26) than in wild-type.

C, Hypocotyl length measurement shows wild-type and *Atlpk2β* overexpression lines (OX-9 and OX-26) have similar hypocotyl length under light-grown conditions, however, *Atlpk2β* overexpression lines have much longer hypocotyls than those of wild-type under dark-grown conditions. Error bars represent SE (n>12).

**Figure 5.** *Atlpk2β* overexpression plants display altered IAA responses. Error bars represent SE (n>12), and statistical analysis indicated significant difference (P<0.01).

A, Effect of IAA on root elongation. The *Atlpk2β* overexpression plants have much longer primary root and show much resistance to the inhibition of IAA on root elongation.

B, Lateral root formation in response to IAA. *Atlpk2β* overexpression plants show less increase in root density than wild-type.

In A and B, typical plant phenotypes are shown on the right.

**Figure 6.** RT-PCR analysis of auxin related genes. One-week-old seedlings were used for all experiments.

A, Wild-type seedlings were treated with 40μM IAA for 0, 0.5, 1, 2 h, respectively. *Atlpk2β* expression was induced by IAA after 0.5 h, while *Atlpk2α* expression was not induced by IAA.

B, Compared with wild-type, CYP83B1 expression was decreased and PIN4 expression was increased in *Atlpk2β*-overexpressing plants (OX-26), whereas, expression of NIT1, TRP, YUCCA, CYP79B2, PIN1, PIN2, PIN3, and PIN7 was not changed.

C, Transcript levels of MAX4 and SPS decreased in *Atlpk2β*-overexpressing plants (OX-26), while levels of AXR1 was not changed.

D, Transcript levels of *Atlpk2β* was not changed in wild-type (Col), axr1 and max4.
Supplemental Figure 1. GUS activity in root tips of ten-day-old seedlings.

A, DR5::GUS
B, Atipk2β::GUS
C, Wild-type

Supplemental Figure 2. DR5::GUS expression patterns in 14-day-old seedlings. (Wild-type and ipk2β mutant)
Table1. Growth and axillary shoots of wild-type and Atlpk2β overexpression plants.

|                                | Wild-type | Overexpression plants |
|--------------------------------|-----------|-----------------------|
| 8.2±0.8 am)                   | 21.1±1.2  | 31.6±1.1\(^a\)       |
| Number of total branches      | 13.4±0.6  | 37.9±2.5\(^a\)       |
| Number of first order branches| 5.1±0.3   | 8.2±0.8\(^a\)        |
| Number of second order branches| 8.3±0.3  | 29.7±1.7\(^a\)       |
| Number of total nodes         | 21.6±5.6  | 51.5±2.3\(^a\)       |
| Branch/node ratio             | 0.6±0.1   | 0.7±0.1               |
| Number of rosette branches    | 1.3±0.2   | 3.7±0.8\(^a\)        |
| Number of cauline branches    | 3.8±0.1   | 4.5±0.5               |

\(^a\) indicates that the mean values are significantly different from those of wild-type (P<0.05)(n=5).

Data are mean±standard deviation.
