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ORIGINAL RESEARCH

# *OsFCA* Transcripts Show More Complex Alternative Processing Patterns than its *Arabidopsis* Counterparts

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**Abstract** The *FCA* gene, which is a component of the autonomous pathway that regulates flowering time, is an important example of how alternative processing can control plant development. We have previously characterized the *FCA* homolog, *OsFCA*, from a japonica-type rice cultivar and demonstrated that the polyadenylation site within intron 3, which can generate non-functional *FCA-β*, was conserved in rice. In this study, we detected five alternatively processed variants of *OsFCA* pre-mRNA, four of which were equivalents of *FCA-α*, *-β*, *-γ*, and *-δ*, in japonica-type Korean rice cultivars. The fifth transcript, referred to as *OsFCA-ε*, was similar to *OsFCA-γ*, except a part of the *OsFCA* intron 16 was retained. Unlike the FCA-γ protein, the OsFCA-γ protein contains a glycine-rich region at its N-terminus. We detected the OsFCA transcripts missing the region encoding the glycine-rich domain in the indica-type rice, but not in the japonica-type rice. We also found that the *OsFCA-δ* and *OsFCA-ε* transcripts were expressed in almost all of the different tissue types examined. Taken together, these results indicate that the alternative processing of the *OsFCA* transcript is more complex than its *Arabidopsis* counterpart.

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## Introduction

Differential RNA processing can cause an increase in transcriptome diversity and the alternatively spliced transcripts may produce distinct protein isoforms thus contributing to proteome diversity in higher eukaryotes (Reddy 2007). This RNA processing affects many cellular events, such as the deletion or modification of proteins and changes in RNA stability, and thus plays an important mechanism in regulating gene expression at the posttranscriptional level. Although recent global analyses of *Arabidopsis* and rice genomes have revealed that alternative RNA splicing is a prevalent phenomenon in plants, data related to the functional significance of alternatively processed plant mRNAs are still limited (Kalyna et al. 2006). One example of this is the alternative processing of the *FCA* pre-mRNA, which has been shown to limit the amount of functional FCA protein in a temporal and spatial manner to control *Arabidopsis* flowering time (Macknight et al. 2002; Quesada et al. 2003; Simpson et al. 2003). The *FCA* gene, which is a member of the autonomous pathway that controls flowering time, encodes an RNA binding protein that has a protein–protein interaction domain (Macknight et al. 1997; Oh and Lee 2007). *FCA* pre-mRNA is alternatively processed, resulting in the generation of four different transcripts (α, β, γ, and δ). It has been shown that the full-length FCA protein (FCA-γ) can promote premature cleavage and polyadenylation within intron 3 of its own pre-mRNA so that a truncated transcript, *FCA-β*, could be produced at the expense of the fully spliced transcript, *FCA-γ* (Quesada et al. 2003). This negative feedback autoregulation of *FCA*

expression is developmentally regulated to control flowering time (Macknight et al. 2002). The alternative processing of *FCA* intron 3 is also conserved in other dicots such as *Brassica napus* and pea (Macknight et al. 2002). This suggests that the *FCA* gene may be an important model of alternative RNA processing for developmental control in higher plants.

We have previously demonstrated that the alternative processing of the *FCA* gene was evolutionarily conserved and that the transcripts (OsFCA-$\alpha$, -$\beta$, and -$\gamma$) equivalent to *FCA*-$\alpha$, -$\beta$, and -$\gamma$ could be detected in japonica-type Korean rice (Lee et al. 2005). We also noticed that several indica-type rice mRNAs (*rFCA-1*, *-2*, *-3*, and *-4*), which were similar to the *OsFCA* transcripts, were in the public sequence database (Lee et al 2005). These indica-type rice transcripts were later renamed as *OsFCA-1*, *-2*, *-3*, and *-4* (Du et al. 2006). The *OsFCA-1* mRNA (accession no. AY274928) encodes an OsFCA-γ protein that results from the splicing of all intron sequences. The *OsFCA-2* mRNA (AY311344) encodes a truncated protein that lacks the WW domain. This truncation results from an alternative splicing event that occurs around intron 13, which is similar to the case of the OsFCA-δ specific transcript (Winichayakul et al. 2005). The *OsFCA-3* (AY311343) and *-4* (AY331574) mRNAs encode for proteins that have two RNA binding domains (RRM) and a WW domain, but due to alternative splicing, these proteins lack a glycine-rich region at their N-termini (Lee et al. 2005). Furthermore, the OsFCA-4 mRNA encodes a protein that lacks a domain similar to the AtSWI3B domain at its C-terminus (Sarnowski et al. 2002) due to another form of alternative splicing. The three transcripts equivalent to *FCA*-$\beta$, -$\gamma$, and -$\delta$ were also identified in ryegrass (Winichayakul et al. 2005).

In this study, we report on the complex alternative processing patterns that occur in *OsFCA* pre-mRNA and the expression patterns of the alternatively spliced *OsFCA* transcripts. As in *Arabidopsis*, the *OsFCA* gene generated the $\alpha$, $\beta$, $\gamma$, and $\delta$ transcripts; however, the alternative processing of the *OsFCA* pre-mRNA appears to be more complex. In addition, alternatively spliced *OsFCA* transcripts were ubiquitously expressed in almost all of the different tissue types examined.

## Materials and Methods

### Plant Material and Growth Conditions

Japonica- and indica-type Korean rice cultivars (*Oryza sativa* L., cv. Ilpoom and *O. sativa* L., cv. Taebyeok, respectively) were used in this study. In order to examine the expression pattern of alternatively spliced *OsFCA* transcripts, various types of rice plant tissue (leaves, roots, stems, calli, and panicles) were obtained, as described previously (Lee et al. 2005).

### RNA Isolation and RT-PCR

Total RNA was extracted from a variety of different tissue types using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the first-strand of complementary DNA (cDNA) was generated from 1 μg of total RNA in accordance with the manufacturer's instructions. To specifically detect alternatively spliced *OsFCA* transcripts, various combinations of the following oligonucleotides were used as the processed transcript-specific primer sets: primer 1 (5′-TCCTCTCC TCTCTCGTGCT-3′), primer 2 (5′-TGTAGACATCTTC CACATGACC-3′), primer 3 (5′-ATAGGTTATCTGGTCA GCTTCCTG-3′), primer 4 (5′-TTCCGAGTAACAGC TTACCAATTT-3′), primer 5 (5′-AGATTGGATGCCC TAAATGTTACCGA-3′), and primer 6 (5′-CACCAATGTG TACGCGGTTT-3′). Each PCR product was cloned and sequenced. To detect the expression levels of alternatively spliced *OsFCA* transcripts, semi-quantitative RT-PCR was performed, as described previously (Lee et al. 2005).

## Results and Discussion

We have previously demonstrated that the alternative processing of the *FCA* gene was evolutionarily conserved and that the transcripts (OsFCA-$\alpha$, -$\beta$, and -$\gamma$) equivalent to *FCA*-$\alpha$, -$\beta$, and -$\gamma$ could be detected in japonica-type Korean rice (Lee et al. 2005). Some other alternatively processed *OsFCA* transcripts have also been identified in indica-type rice (Du et al. 2006; Lee et al. 2005; Winichayakul et al. 2005). In order to determine whether these indica-type *OsFCA* transcripts also exist in japonica-type rice, we performed RT-PCR using primers designed to detect the specific regions of the transcripts (Figs. 1, 2 and 3). First, we attempted to detect the *OsFCA-δ*- and *OsFCA-4*-specific PCR products using primers 3 and 4 from a japonica-type Korean cultivar (Ilpoom bye; Fig. 1a). In the *OsFCA-δ* transcript, intron 13 (116 bp) was retained; thus, its PCR product should be larger than that for the *OsFCA-γ* transcript. However, in the *OsFCA-4* transcript, a part (22-mer) of the intron 16 sequence was retained, which should result in a PCR product that is similar to the *OsFCA-γ* transcript (Fig. 1). Sequence analysis of the RT-PCR products revealed the following: In the larger band (indicated with the letter *a* in Fig. 1a), the PCR product retained intron 13, as was expected for the *OsFCA-δ* transcript of the indica-type rice (Winichayakul et al. 2005). In the smaller band (indicated with the letter *b* in Fig. 1a), there were two PCR products representing the

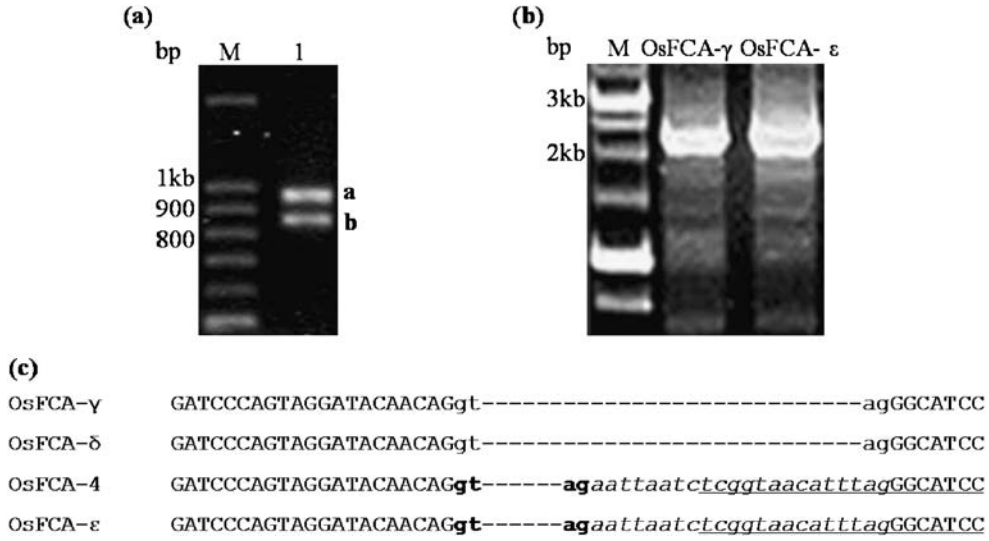


**Fig. 1** Alternatively spliced *OsFCA* transcripts from japonica-type rice. **a** Image of a gel that displays the RT-PCR products for the *OsFCA-γ* and *OsFCA-δ* transcripts that were amplified from a japonica-type Korean cultivar (Ilpoom bye). The *OsFCA-γ* and *OsFCA-4* transcript-specific RT-PCR products were indicated with the letter *b*. Intron 13 (116 bp) was retained in the *OsFCA-δ* transcript-specific RT-PCR products (indicated with the letter *a*). Primer sets 3 and 4 (shown in Fig. 3) were used to amplify these transcripts. *M* indicates the 100-bp size marker. **b** Image of a gel that displays the *OsFCA-γ* and *OsFCA-ε* transcript-specific RT-PCR products. Primer sets 1 and 4 (shown in Fig. 3) were first used to amplify the PCR products corresponding to these transcripts (*OsFCA-γ* lane). Then, nested PCR was performed with primer sets 1 and 5 (shown in Fig. 3) to specifically amplify the *OsFCA-ε* transcript (OsFCA-ε lane). *M* indicates the 1-kb size marker. **c** Alignment of the *OsFCA* transcript sequences corresponding to a region of intron 16. The intron sequences were shown with *small letters* and *hyphens*. The *OsFCA-ε* and *-4* transcripts were generated through the use of new GT-AG splice sites (shown in *bold*) within intron 16. The additional 22 nucleotides in the intron 16 sequence retained in the *OsFCA-ε* and *-4* transcripts are indicated in *italics*. A part of the primer 5 sequence was *underlined*

expected *OsFCA-γ* transcript and the *OsFCA-4* transcript. These results indicate that the japonica-type rice may also contain the *OsFCA-δ* and *OsFCA-4* transcript.

In order to confirm the existence of the *OsFCA-3* and *-4* transcripts in the japonica-type rice, we conducted RT-PCR with primers 1 and 2. However, we were only able to detect them in the indica-type Korean cultivar (Taebyeok bye; Fig. 2). Based on these results, we investigated the nature of the transcript that retained the 22-mer of intron 16, which we believed was the *OsFCA-4* transcript. The initial attempt to isolate the full-length transcript containing the 22-mer using primers 1 and 4 was unsuccessful. Subsequent analysis revealed that all sequenced PCR products were from the *OsFCA-γ* transcript (data not shown). Therefore, we decided to perform a nested PCR using primers(primer 5) that were designed to span the 22-mer and exon 17 (Fig. 1c). Using this method, we were able to detect a unique transcript (named as *OsFCA-ε*) that retained the 22-mer of intron 16 and the glycine-rich region (Fig. 1b, c).

In addition, we found that the transcripts detected from the indica-type Korean cultivar were spliced at sites different from those of the *OsFCA-3* and *-4* transcripts, although they were still missing most of the glycine-rich regions (Fig. 2). We examined the leaves and roots of the indica-type Korean cultivar for the presence of these transcripts. From this analysis, we detected two transcripts (TBL1 and TBL2) from the leaves and one transcript (TBR1) from the roots (Fig. 2). They all were spliced at non-canonical splice sites and their spliced-out regions were shorter than those of the *OsFCA-3* and *-4* transcripts. Only the TBR1 transcript, if translated, would produce a OsFCA protein that was missing the glycine-rich region. The TBL1 transcript could not produce a truncated OsFCA protein because the splicing process caused a translational frame shift in the coding region. The TBL2 transcript could also not produce a functional OsFCA protein because the initiation codon was deficient due to splicing and the second methionine codon was located in the middle of the RNA binding domain of the OsFCA protein. These results suggest that alternative splicing may not be tightly regulated in the *OsFCA-3* and *-4* transcripts of the indica-type rice. Since the *OsFCA-3* and *-4*-type transcripts were not detectable in the japonica-type rice, these transcripts may not be conserved between these two rice subspecies. The complex alternative splicing patterns of the *OsFCA* gene were summarized in Fig. 3.

Since the relative amount of *OsFCA* transcripts ($\alpha$, $\beta$, $\gamma$, $\delta$) was already known (Du et al. 2006; Lee et. al. 2005; Winichayakul et al. 2005), we examined the temporal and

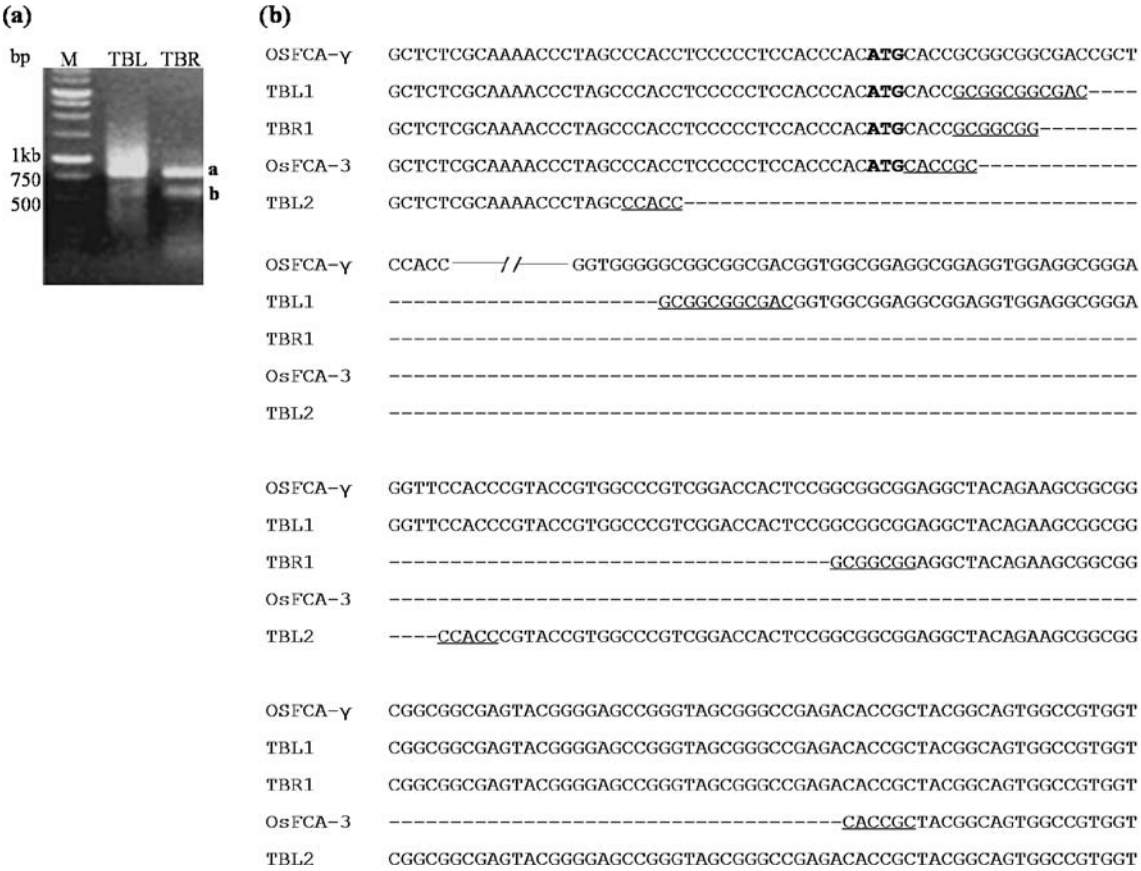


**Fig. 2** Alternatively spliced *OsFCA* transcripts from indica-type rice. **a** Image of a gel that displays the *OsFCA-γ* and *OsFCA-3/-4* transcript-specific RT-PCR products. The *OsFCA-3/-4* transcript-specific RT-PCR products were obtained from the indica-type Korean rice cultivar, Taebyeok bye. Primer sets 1 and 2 (shown in Fig. 3) were used to amplify the PCR products corresponding to these transcripts. The *TBL* and *TBR* lanes show the PCR products amplified from the leaves and roots of Taebyeok bye, respectively. The letters *a* and *b* indicate the *OsFCA-γ* and *OsFCA-3/-4* transcript-specific RT-PCR products, respectively. The signal indicated with the letter *b* in the TBL lane appears weaker. *M* indicates the 1-kb size marker. **b** Alignment of the *OsFCA* transcript sequences corresponding to a region of exon 1. The glycine-rich region of exon 1 was spliced out as an intron in the *OsFCA-3* transcript. The intron sequences deleted in the indica-type *OsFCA* transcripts (TBL1, TBL2, TBR1, and *OsFCA-3*) are indicated with *hyphens*. Duplicated sequences are *underlined*, since any combination of splice sites within them was possible. All splice sites were non-canonical

spatial expression patterns of the *OsFCA-δ* and *OsFCA-ε* transcripts (Fig. 4). The appropriate primer sets were used to amplify the *OsFCA-δ* and *OsFCA-ε* transcripts. These transcripts were detected in all of the tissue types tested; however, both had lower expression levels in roots. These results are similar to the relative expression levels of the indica-type *OsFCA* transcripts found in the leaves and spikelets of Chinese hybrid rice lines (Du et al. 2006). The expression of *OsFCA-δ* remained unaltered during the panicle developmental stages. This is in contrast to the expression of *OsFCA-γ*, which was high in the early stage of panicle development (Lee et al. 2005).

Alternative RNA splicing in plants have some unique features relative to their animal counterparts (Reddy 2007). One unique feature is that intron retention is the most prevalent type of alternative splicing in plants and the rarest type in animals (Kim et al. 2007; Ner-Gaon et al. 2007). An example of this is the retention of intron 3 in the FCA alpha transcript (Macknight et al. 1997). Immunodetection of endogenous FCA proteins revealed that only the protein for the *FCA* gamma transcript was detectable, suggesting that the alternatively spliced transcripts ($\alpha$, $\beta$, $\delta$) may not be translated (Quesada et al. 2003). The loss-of-function *fca-1* and *fca-4* mutants contained less beta and more alpha transcripts than the wild type. Therefore, the alpha and beta transcripts may be the result of the negative regulation of *FCA* expression at the level of splicing versus polyadenylation at intron 3 (Quesada et al. 2003). The *FCA* delta transcript exists at a much lower level and its function is not yet known (Macknight et al. 1997).

Although alternative splicing of FCA pre-mRNA was well conserved in the rice counterpart, there were some differences. We found that the exon 1 of the *OsFCA* gamma transcript could contain an intronic sequence only in indica-

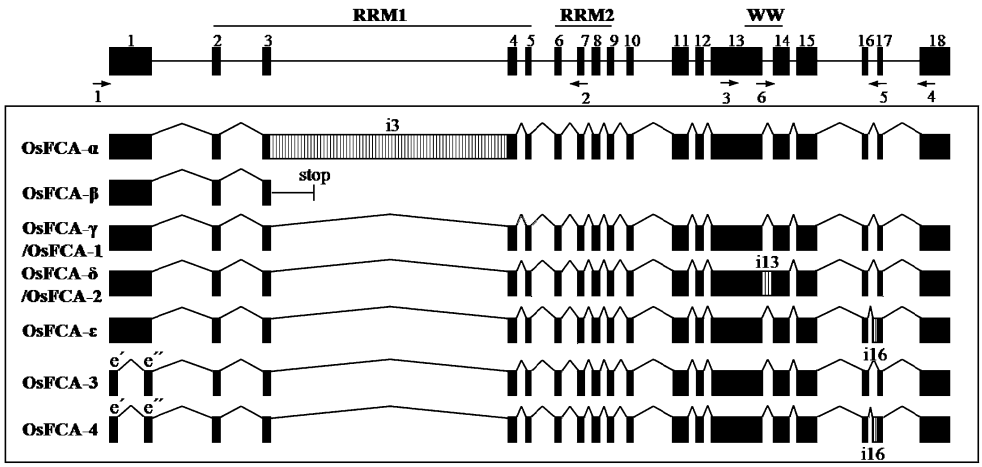


**Fig. 3** Schematic illustration of alternatively spliced *OsFCA* transcripts. The black boxes indicate exons and the lines indicate introns. The slashed boxes correspond to the introns retained in the alternative transcripts. The glycine-rich region of the exon 1 was spliced out as intron in *OsFCA-3* and *-4* transcripts in indica-type rice. The approximate locations of the two RRM and the WW domain are indicated. The locations of primers used to amplify various alternative transcripts are indicated with arrows

type rice; thus, it is possible that the *OsFCA* gamma transcript was the product of intron retention in indica-type rice. However, this alternative splicing occurred at splicing sites that were different from those previously known, suggesting that this process was not tightly regulated (Fig. 2). Therefore, these transcripts might be the result of unsuccessful splicing of the *OsFCA* pre-mRNA. In this study, we also found a new *OsFCA* transcript, epsilon, in which an alternative 3′ splice site was used in the splicing of intron 16, as compared to the gamma transcript. This transcript would produce a gamma protein that had a few amino acid residues missing from its C-terminus. To detect the *OsFCA-ε* transcript, we had to use nested PCR because regular RT-PCR only detected the *OsFCA-γ* transcript (Fig. 2). This indicates that the abundance of the *OsFCA-ε* transcript is much lower than that of the *OsFCA-γ* transcript.

Alternative splicing has been thought to be a major pathway for expanding the functional diversity of the human proteome. However, there have been two contradictory reports in regards to this hypothesis. One study claimed that most of the alternative gene products in humans would not result in functional proteins because their structure and function would be quite different from their constitutively spliced counterparts (Tress et al. 2007). The other study arrived at the opposite conclusion in terms of the production of functional proteins from alternative gene products (Birzele et al. 2008). The discrepancy between these two views will eventually be resolved through extensive human proteome studies.

Compared to the human genome, alternative splicing occurs at a much lower incidence (about 20%) in Arabidopsis and rice genomes (Wang and Brendel 2006). Furthermore, the most common type of alternative splicing is intron retention, which often introduces premature translational termination. It has been suggested that alternative splicing in plants may play a more important role in posttranscriptional regulation than expanding the proteome (Campbell et al. 2006). In order to explain the biological meaning of the complex alternative processing

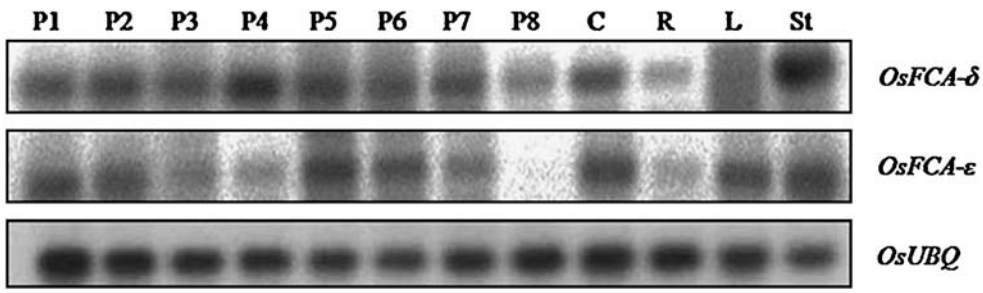


**Fig. 4** Spatial and temporal expression patterns of the *OsFCA-δ* and *OsFCA-ε* transcripts. Semi-quantitative RT-PCR was performed to measure the relative abundance of the *OsFCA-δ* and *OsFCA-ε* transcripts. The primer sets 4/6 and 3/5 (shown in Fig. 3) were used to amplify *OsFCA-δ*, and *OsFCA-ε*, respectively. Primers 6 and 5 were designed to detect the retained intronic sequences of the *OsFCA-δ* or *OsFCA-ε* transcripts, respectively. The *lanes* show panicles of eight developmental stages (P1 to P8), calli (*C*), roots (*R*), leaves (*L*), and stems (*St*), which were classified as described in Lee et al. (2005). The *OsUBQ* gene was employed as an internal control

patterns of the *OsFCA* pre-mRNA, we first have to know the function of *OsFCA*. Therefore, further investigation is needed to determine whether these alternatively processed transcripts are the negative consequences of regulating *OsFCA* expression or if they perform unknown functions.

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