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Full length Research paper

Comparative studies between contrasting genotypes involved in rice seed germination using cDNA-AFLP technique

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Rice is a model for many genomic studies among crop species. The adaptation to different crop environments is partly the result of traditional breeding combined with molecular assisted selection. Comparative studies between contrasting genotypes can be performed efficiently by the cDNA-AFLP technique, allowing the identification of genes involved in the expression of the target trait. The objective of this study was to identify differentially expressed genes in cultivars contrasting for the character germination at low temperatures. The genotypes used were BRS-Firmeza (Tolerant) and SCSBRS Tio-Taka (Sensitive). Seeds of the two cultivars were sown at 13 and 25°C, respectively. Total RNA was extracted from embryos and cDNAs were synthesized. The cDNAs were analyzed by AFLP (Amplified Fragments Length Polymorphisms) to identify differentially expressed sequences between BRS-Firmeza and SCSBRS Tio-Taka varieties. Four polymorphic fragments were present at 13°C IN BRS-Firmeza molecular profile and absent in SCSBRS Tio-Taka. It is concluded that the technique of AFLP on cDNA as template is efficient to produce and identify fragments of differentially expressed genes in rice during germination under low temperatures.

Key words: *Oryza sativa,* seeds, cDNA-AFLP (cDNA amplified fragment length polymorphism).

INTRODUCTION

Rice (*Oryza sativa* L.), species grown in all continents, has a great social and economical importance, besides being considered a model plant for molecular studies, especially among monocotyledons (Bennetzen and Freeling, 1993; Gale and Devos, 1998; Bennetzen, 2002; Sguarezi et al., 2003).

Rice is part of 66% of the global population diet, especially in the agricultural world with a production of 590 million tons and approximately 150 million hectares annually (Embrapa, 2009). This makes rice the species with the greatest potential to fight world hunger, due to its versatility and adaptability to different soils and climates.

The Rio Grande do Sul state is responsible for more than half of Brazilian production, with little more than a million hectares cultivated. The crop is the main agricultural economic source in the south area of Rio Grande do Sul (CONAB, 2009). Low temperatures represent one of the environmental stresses that most adversely affect plant growth (Xiong et al., 2002; Cui et al., 2005) affecting mostly at the reproductive stage, followed by germination and emergence (Freitas, 2005; Amaral, 2010).

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The breeding methods allow breeders to explore more efficiently the genetic resources. Approximately 50% of productivity increases in cultivated species are due to genetic improvement, besides the undeniable reduction in production costs and the incorporation of new production areas owing to the introgression of suitable cultivars (Ramalho, 2004; Neto et al., 2008). Part of this success was due to modern genomic techniques, such as knowledge of many species and assisted breeding by molecular markers (Zimmer et al., 2005). The complete sequencing of rice genome was announced in 2004, creating expectation to identifying genes that are important in breeding and which contributes to increase productivity of this crop and to the best adaptation in regions with conditions considered unfavorable for crop establishment (IRGSP, 2005).

In this context, researches are conducted with molecular genetic approaches using contrasting genotypes for a target trait. Among the promising techniques, one can highlight the isolation of fragments from differentially expressed cDNAs - cDNA / AFLP (cDNA amplified fragment length polymorphism) (Mao et al., 2004), which allows the identification and isolation of gene fragments related to the existing contrasts between genotypes and developmental stages. In this study, the character studied is the germination performance and emergence at low temperatures.

Among the various molecular markers techniques, the AFLP (amplified fragment length polymorphism) has been used, mainly due to the ability to assess the variability at genome level, detecting a large number of fragments per reaction. Thus allowing a wide and simultaneous sampling, which makes the technique more specific and with significant reproducibility (Spooner et al., 2005).

The aim of this study was to identify the presence of differentially expressed genes in contrasting cultivars for the germination trait when induced to low temperatures stress.

MATERIALS AND METHODS

This work was conducted at the Seed Analysis and Bioseeds Laboratory of the Federal University of Pelotas (UFPel).

Plant material

The genotypes BRS Firmeza and SCSBRS Tio Taka, tolerant and sensitive to low temperatures, respectively, were used. The seeds of both genotypes were subjected to germination tests at temperatures of 13 and 25°C to obtain embryos at the same developmental stage. The embryos were collected at the end of Phase I and during Phase II, as "Three Phase Standard" proposed by Bewley and Black (1978) and adapted by Marcos (2005).

Plant tissue obtaining

Seeds of the cultivars SCSBRS Tio Taka and BRS "Firmeza" were sown on germitest paper and moistened with distilled water in ratio of 2.5 times the dry weight of the paper and placed in gerbox. There were sown 100 seeds of each genotype and placed in germination chamber at 13 and 25°C.

The embryos were collected at the first, second and third day after sowing when 25° C were used, whereas at 13°C, the embryo collection was carried out at fourth, eighth, eleventh and fourteenth day after sowing. The procedure was necessary because low temperatures slow down enzymatic activity and consequently the germination process.

Embryo isolation

For this study, 45 embryos at 25°C were isolated (20, 15 and 10 seeds at the first, second and third sampling, respectively) and 65 embryos at 13°C (20, 20, 15 and 10 seeds at the first, second, third and fourth collection, respectively) for each cultivar. With the aid of a blade, the embryo was isolated, taking care to keep the plant tissue clean, in other words, without any trace of endosperm or any other plant tissue that was not embryo, which was immediately placed on ice. The procedure was performed with sterilized material, treated with ultrapure water containing 0.01% diethyl pyrocarbonate (DEPC) and in aseptic environment, free from RNAses and external contamination of RNAs. After isolation, the embryos were placed in sterile tubes, identified and stored in a freezer (-80° C) until the total RNA extraction.

Extraction of total RNA

All procedures involving the collection and subsequent handling of the total RNA were performed using aseptic environment using laminar flow and / or exhaustion chapel, using sterile equipment and all reagents were prepared with ultrapure water treated with DEPC at 0.01%.

Total RNA was isolated using the Reagent Pure Link Plant RNA (Invitrogen) according to the manufacturer's recommended use. For extraction, the embryos, from each collection were grounded in liquid nitrogen and added the Reagent Pure Link Plant RNA. 5 M NaCl and chloroform was used to facilitate the separation of aqueous and organic phases and removal of contaminants. In the last step, the total RNA present in the aqueous phase was precipitated using isopropanol. The total RNA pellet was dissolved in 20 µl of treated ultrapure water with DEPC at 0.01% and stored in freezer at -80°C until the cDNA synthesis.

The integrity evaluation of RNA samples extracted and the estimate of their quantity were obtained by visualization in agarose gel. The electrophoresis was performed using Tris / Borate / EDTA (TBE) 1X, according to Sambrook et al. (1989). 3 ∝l of RNA were prepared with 2 ∝l of load buffer and applied on agarose gel at 1% in TBE 1X, stained with ethidium bromide at concentration of 0.2 µg/ml. After electrophoresis, gels were examined in ultraviolet light and digital images were obtained.

cDNA synthesis

After extraction of total RNA, bulks were made for each genotype and germination temperature, using 3 µl of RNA in water with diamethyl pyro carbonate (DEPC) from each sampling at 25ºC, totaling 9 and 2.5 µl of each sample at 13ºC, and totaling 10 µl.

For the synthesis of double-stranded cDNA, the SuperScript µ Double-Stranded cDNA Synthesis Kit (Invitrogen) was used, according to manufacturer's recommendations. This procedure starts with the synthesis of the first strand using oligo dT primers, dithiothreitol (DTT), mix of phosphate ribonucleotides deoxy

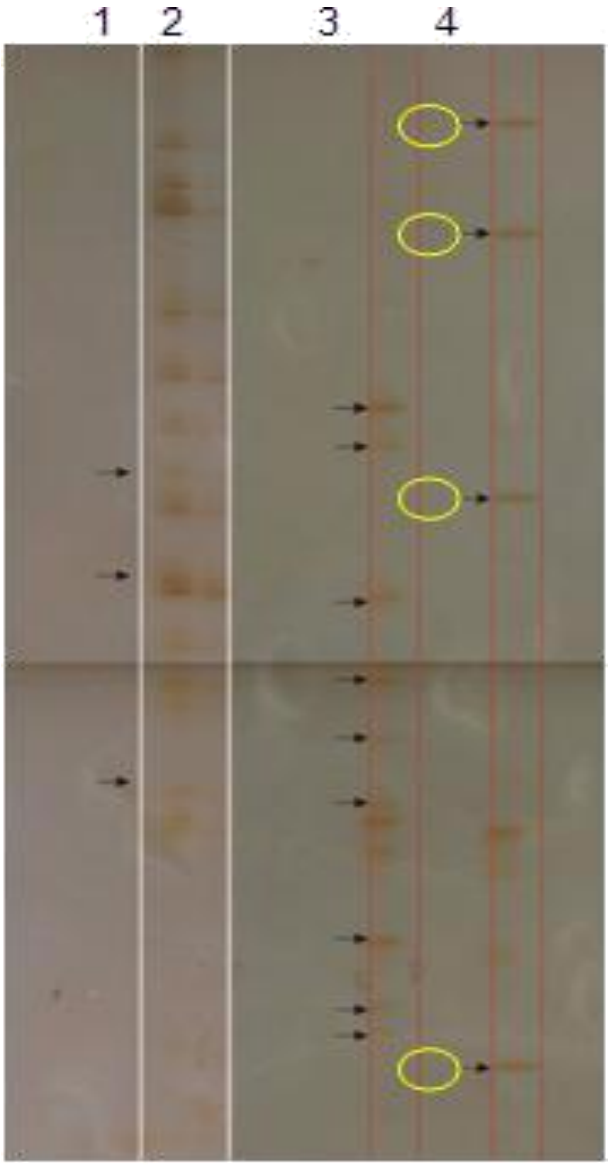

Figure 1. Separation of fragments by the technique of cDNA-AFLP in polyacrylamide gel 6%. Groove: 1 - SCSBRS Tio Taka at 25°C, 2 - SCSBRS Tio Taka at 13°C, E-ACC/M-CAT primers. Groove: 3 - BRS Firmeza at 25°C, 4 - BRS Firmeza at 13°C, E-AAG/M-CTT primers.

(dNTPs) and the enzyme reverse transcriptase. The subsequent synthesis of the second strand of cDNA was performed in a reaction containing a dNTP mix, DNA ligase enzymes, DNA polymerase and RNase, and after that, with T4 DNA polymerase. For cDNA isolation, 0.5 M EDTA, phenol: chloroform: isoamyl alcohol 25:24:1, ammonium acetate 7.5 M and ethanol in order to obtain cDNA intact and free from contamination. The pellet obtained was dissolved in 6 µl of DEPC water.

To measure the efficiency of cDNA yield and its quality, fragments were electrophoresed in agarose gel 1% on 1X TBE, according to SAMBROOK et al. (1989). One microliter of cDNA was applied in the gel, stained with ethidium bromide (0.2 µg/ml), examined by ultraviolet light and digital images were obtained.

AFLP analysis

To implement this technique, the AFLP Starter Primer Kit (Invitrogen) was used following manufacturer's recommendations. The cDNA digestion steps were done with restriction enzymes (EcoRI I and Mse I) and binding of specific adapters to the terminals of the cDNA fragments generated by cleavage, which have complementary sequences to primers used in the PCR reaction. After this, a pre-amplification of restriction fragments with only one selective base in primer (EcoRI I = A and Mse I + C) was performed. The product of the pre-amplification was used as template DNA for selective amplification reactions.Ten primers were tested: E-AAC/M-CAA, E-AAG/M-CAC, E-ACA/M-CAG, E-ACC/M-CAT, E-ACG/M-CTA, E-ACT / M -CTC, E-AGC/M-CTG, E-AGG/M-CTT, E-AGC/M-CAC and E-AAG/M-CTT.

Data analysis and results interpretation

The visualization of differentially expressed cDNA fragments was performed by electrophoresis on 6% polyacrylamide gel and silver-stained according to methodology described by Beidler et al. (1982).

The fragments differentially expressed were compared between the different genotypes and between the two temperatures (presence or absence in certain molecular weight of fragments).

The molecular profiles analysis was performed visually by identifying polymorphic bands, in other words, present in one genotype and absent in other, or present at the same temperature and absent in another in the same genotype.

RESULTS AND DISCUSSION

In the selective amplification step, from ten combinations of tested primers, six showed no amplification and in four, cDNA polymorphic fragments were observed in relation to temperature and genotypes. The primers that showed amplification were E-ACC/M-CAT, E-ACG/M-CTA, E-AAG/M-CTT and E-AGC/M-CTG. The comparison between the genotype BRS Firmeza and SCSBRS Tio Taka painted out that both the seeds germinated at 25°C, as at 13°C showed differential expression of genes, and the 10 primer combinations tested, four amplified, resulting in 23 polymorphic fragments, being 18 at 25ºC, and polymorphic bands at 13ºC (Figures 1 and 2).

In germination at 25°C, using a combination of primers E-ACC/M-CAT two polymorphic fragments were identified, with a band presence on the genotype SCSBRS Tio Taka, indicating that two possible genes were expressed in this genotype at 25ºC, not occurring the same when the temperature was 13ºC. In genotype BRS Firmeza, 13 polymorphic fragments were found, nine present in at a temperature of 25ºC and four at a temperature of 13°C, indicating that four possible genes were expressed at this temperature, resulting from the combination of primers E-AAG/M-CTT.

Comparing the germination of both genotypes at 25°C between the primers E-ACC/M-CAT and E-AAG/M-CTT, it was observed in genotype SCSBRS Tio Taka, the expression of two possible genes, whereas in the BRS Firmeza there were expression of nine. As for

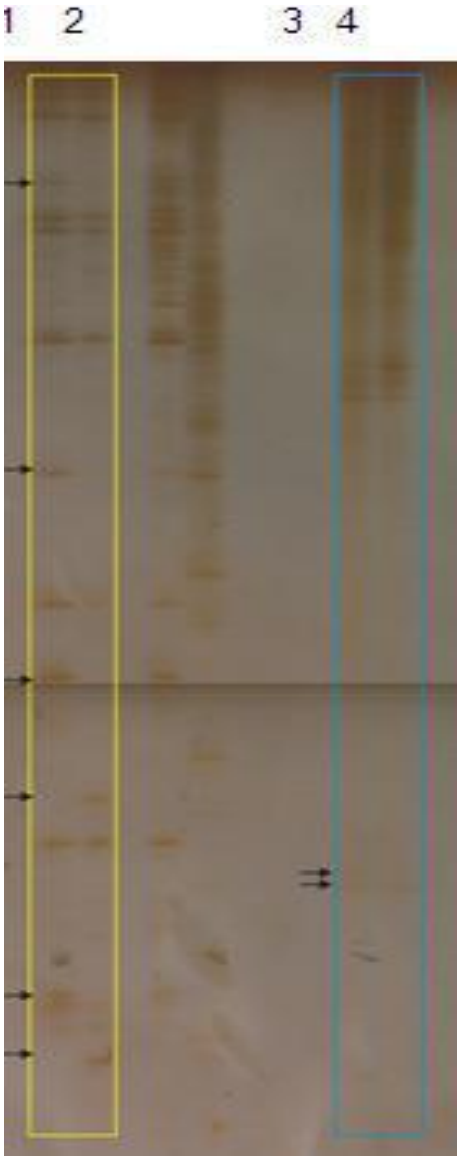

Figure 2. Separation of fragments by the technique of cDNA-AFLP in polyacrylamide gel 6%. Groove: 1 - SCSBRS Tio Taka at 25°C, 2 - SCSBRS Tio Taka at 13°C, *E*-ACG/*M*-CTA primers. Groove: 3 - SCSBRS Tio Taka at 25°C, 4 - SCSBRS Tio Taka at 13°C, *E*-AGC/*M*-CTG primers.

germination at 13ºC, as shown in Figure 1, no polymorphic band was expressed in SCSBRS Tio Taka, a sensitive cultivar, when exposed to cold stress to germinate in combination of primers E-ACC/M-CAT, unlike the BRS Firmeza, a resistant cultivar, which when exposed to this stress, it expressed four bands in E-AAG/M-CTT combination.Using a combination of primers E-ACG/M-CTA, in germination at 25°C, it identified five polymorphic fragments in SCSBRS Tio Taka and one polymorphic fragment at 13°C. However, in the combination of primers E-AGC/M-CTG, two polymorphic fragments were all expressed at 25°C.

Genotype SCSBRS Tio Taka when compared in temperature of 25°C, for the combination of primer E-ACG/M-CTA, it expressed five polymorphic bands, while in E-AGC/M-CTG primers, two were expressed. However, when comparing at the temperature of 13°C, it expressed a band on the combination of E-ACG/M-CTA primers and none for the E-AGC/M-CTG combination. This polymorphic band expressed in E-ACG/M-CTA primers, at 13ºC, may be the expression of an enzyme inhibition, and not induction of germination.

Considering the cultivars SCSBRS Tio Taka and BRS Firmeza, sensitive and tolerant to cold, respectively, polymorphisms were observed on BRS Firmeza, in both temperatures, and none for SCSBRS Tio Taka at 13ºC (Figure 1). The selective amplification produced fragments differentially expressed, suggesting that several genes are still expressed in the BRS Firmeza when germination occurs at low temperatures, these genes are probably related to its tolerance to low temperature.

In general, the pattern of polymorphic bands most frequently observed in this study was that in which the cultivar SCSBRS Tio Taka did not express fragments at 13°C, while in genotype BRS Firmeza, there was expression (Figure 1). This behavior can be attributed to the fact of cultivar SCSBRS Tio Taka being more sensitive to low temperatures, where it does not express the genes in the germination stage.

The most important level of regulation is the beginning of transcription. From certain signs, regulatory sequences are recognized by protein factors, allowing the activation of a gene or group of genes, with transcription (Zaha et al., 2003). On the other hand, several internal signals are required to coordinate gene expression during development and to enable the plant to respond to environmental signals. Such internal flags agents as well as external, typically promote their effects by means of biochemical reactions sequences, called “routes of signal transduction”, which amplify the original signal and ultimately result in the activation of genes (Taiz and Zeiger, 2004). Therefore, when the germination temperature is in the sub-optimal range, probably, the gene regulation is affected, occurring genetic variation from genotype to genotype, which can be observed when comparing the differential expression of genotypes bands between SCSBRS Tio Taka and BRS Firmeza (Figure 1).The existence of different physiological mechanisms involved in tolerance to cold indicates that during the germination phase, different processes may be acting as mechanisms of tolerance (Cruz and Milach, 2004), resulting in the expression of different genes. Previous studies, report that the response to low temperatures is complex and probably controlled by more than one gene (Cruz and Milach, 2000; Viswanathan and Zhu, 2002; Andaya and Mackill, 2003). Since the BRS Firmeza is

described as moderately tolerant to cold, these fragments can be related to the expression of important genes for this characteristic. The development process of cold tolerance involves the synthesis of several different proteins that accumulate during cold adaptation, as a result of change in gene expression (Guy, 1999).

In rice, Rabbani et al. (2004) identified 36 genes inducible by cold. Per homology, these genes were classified into two groups according to their product: (a) Functional proteins, having probably some role in stress tolerance, such as LEA proteins (Late Embryogenesis Abundant) COR proteins (Cold-Regulated) involved in cold adaptation (many encode hydrolytic polypeptides, which hypothetically play a role in protecting the cells to low temperature, among others); and (b) Regulatory proteins which are protein factors involved in regulating the translation signal and gene expression that probably work in response to stress, and it may be the type finger RINF, finger ZINC, the family of leucine zipper motif, protein cinases, phosphatases and enzymes involved in phospholipid metabolism.

The most intense fragments and with a good resolution were recovered in order to make the reamplification and sequencing, allowing later on the genes identification that do express this characteristic, possibly related to cold tolerance at germination stage. Breeding using molecular tools does differ from conventional breeding by giving more predictable results, with specific genes from a donor to a receiver in a controlled manner. The interaction of standard methods of genetic breeding with strategies and technologies of genomics allows the establishment of new goals for the development of superior cultivars. The field of molecular techniques is important in precision and speed of the breeding processes, besides reducing the time needed to obtain a new variety.

Molecular markers can reveal differences between genotypes efficiently, because they act directly on the genome of the organism, not being influenced by environment and can assist in such selection. However, it still needed the desired genes for this process to be more effective. In this scenario, this work enables the identification of differentially expressed genes in rice cultivars under cold stress.

The identification of differentially expressed genes starts by the sequencing of complementary partial sequences of cDNA, with a subsequent cloning of the complete gene. The function identification and the factors responsible for gene expression constitute the main focus of the "genome" project. The information use of DNA banks and proteins helps to elucidate the function and expression of new genes. From the sequences selected in this study, it is possible to identify proteins encoded by genes. The possible functions of these enzymes will establish relationship with the metabolic pathways to which they belong and their role in tolerance to cold stress, in germination stage of rice. The continuity of studies based on band redeemed, using sequencing and *in silico* analysis in future work may be an important instrument available to a breeder in the transmission of specific genes, in a controlled manner.

Overall, for the study of current knowledge about the influence of cold on germination and early development of rice, one could say that this trait is genetic of complex nature and controlled by several genes. The upside of this type of study lies in the fact that control gene for the trait under study is very complex, being the initial step, a breakthrough to obtain intersections with genotypes carriers of phenotypic differences for these characteristics. From experimental analysis for the characterization of different phenotypic classes, derived from these crosses, it is promising to establish, via molecular markers, which genes are responsible for differences in these phenotypic classes identified.

Conclusions

There are differences in genetic expression for the character germination at low temperatures between BRSGO Firmeza and SCSBRS Tio Taka. It is possible to identify four polymorphic fragments at 13°C in the genotype BRS Firmeza. The technique of cDNA / AFLP is efficient for obtaining and identifying fragments of differentially expressed genes in irrigated rice genotypes during germination under stress of low temperatures.

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