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Short Communication

PCR and sequencing from a single pollen grain

Gitte Petersen*, Bo Johansen and Ole Seberg
Botanical Institute, University of Copenhagen, Gothersgade 140, DK-1123 Copenhagen K, Denmark
*(*author for correspondence)*

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Abstract

In order to eliminate the laborious step of DNA extraction preceding all studies within the field of plant molecular biology we attempted to do PCR amplifications directly on pollen grains. Successful PCR amplification was obtained in reactions including a single pollen grain from *Hordeum vulgare* or *Secale strictum*. Both the plastid gene encoding ribulose-1,5-biphosphate carboxylase/oxygenase (*rbcL*) and the nuclear-encoded internal transcribed spacer regions (ITS) and the 5.8S rDNA region were amplified and sequenced to verify PCR amplification.

It is well known that successful PCR amplifications can be performed on DNA extracted from very small amounts of tissue (e.g. [6]) or highly degraded DNA (e.g. [4]). Previously, we have amplified and sequenced the large subunit of ribulose-1,5-biphosphate carboxylase/oxygenase (Rubisco, *rbcL*) from DNA extracted from pollinia of *Dendrobium secundum* (Blume) Lindl. [5]. As pollen contains relatively high amounts of DNA compared to cell size, we chose to bypass the extraction step to see how little pollen material would be sufficient to amplify *rbcL* and the nuclear-encoded internal transcribed spacers (ITS) including the 5.8S rDNA region. Thus, we attempted to do PCR on just a single pollen grain.

Pollen from *Secale strictum* (J. Presl) J. Presl (syn. *S. montanum* Guss.) (accession number H4545, Turkey: Afyon) and *Hordeum vulgare* L. cv. Bonus were used because grass pollen will burst readily when dispersed in water, releasing cell components into the solution. *Secale* pollen was used for amplifying *rbcL*, and *Hordeum* pollen for the amplification of ITS. Each grass pollen contains three nuclei and numerous plastids. The rDNA array of a typical genome consists of several hundred tandemly repeated copies of the transcribed unit [7] and a single pollen grain typically contains more than a hundred plastids, each with a minimum of one genome (personal observation). Thus, in a single pollen grain numerous copies are present of both *rbcL* and ITS. Pollen grains were transferred using an eyelash attached to a toothpick into microtubes containing 14 μl ddH_2O and 5 μl standard TQ-buffer for PCR (0.67M Tris-HCl pH 8.5, 20 mM $MgCl_2$, 166 mM $(NH_4)_2SO_3$, 0.1 M 2-mercaptoethanol). As a control, no pollen was transferred to one of the tubes. Immediately after the transfer, the tubes were heated to 95 °C for 10 min to destroy all enzymatic activity. The PCR amplification was performed in 50 μl reactions running 30 cycles using the procedure outlined by Saki *et al.* [8].

The remaining reagents were added after heat treatment of the tubes. We used primers Z427 and Z1020r designed by G. Zurawski (DNAX Research Institute, Palo Alto, CA) but slightly reduced in length from the 5′ end to amplify a ca. 600 bp fragment of *rbcL*. The ITS primers ITS-4 and ITS-5 [10] were used to amplify both the ITS regions and the small ribosomal subunit. To verify that the correct genes had been amplified, the double-stranded PCR amplifications were followed by single-strand amplifications securing enough copies of DNA for dideoxy termination sequencing [3, 9]. In the reactions, only one strand of each gene was sequenced using the primers Z427 (sequencing *rbcL*) and ITS-4 (sequencing ITS2 and part of 5.8S), respectively.

The *rbcL* amplification was performed in a series of tubes with different numbers of pollen grains (1, 5, 10, 20, and 50; see Fig. 1). Amplification was successful in runs using 5, 10, 20, and 50 pollen grains and also in four out of five runs with only a single pollen grain. A rough correlation between the number of pollen grains added and the quantity of amplified fragments (vizualized as intensity of the fluorescent bands) is evident. Subsequent comparison of a 130 bp sequence (position 486 to 615 of the *rbcL* gene) obtained from pollen showed complete similarity with the same fragment of another *Secale rbcL* sequence (O. Seberg, in prep.) obtained from leaves. The *Secale* sequence differs from the *Hordeum vulgare rbcL* sequence (GenBank accession number X00630 [11]) at one position within this region.

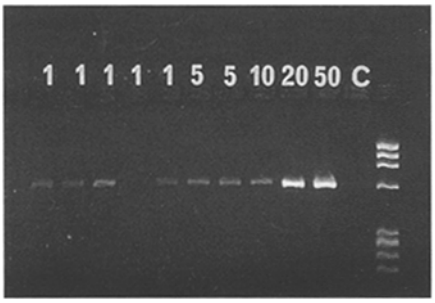

Fig. 1. PCR products of the *rbcL* amplification tested on a 2% agarose gel stained with ethidium bromide. Number of pollen grains added is indicated above each lane. Lane 11 is the control (C). The marker is PhiX174.

The ITS experiment was only performed in tubes with a single pollen grain. Again amplification was successful in four of the five tubes, and the subsequent sequence comparison verified the amplification of the *Hordeum vulgare* ITS fragment. Comparison of part of the amplified segment (293 bp) and the published ITS sequence from *Hordeum vulgare* (GenBank accession number Z11759 [2]) revealed minor differences, but only due to two small compressions in the sequencing gel which did not allow for the reading of one and two basepairs, respectively. A comparison with the published ITS sequence from *Secale strictum* (GenBank accession number Z11760 [1]) showed ca. 92% sequence similarity. Thus, we conclude that we have amplified the correct genes.

Having succeeded amplifying sequences existing in multiple copies per pollen grain, we attempted to amplify a single-copy nuclear gene, Adh_1 (alcohol dehydrogenase 1), present in three copies per pollen grain. However, the results have so far been negative even in reactions including more than a hundred pollen grains. Thus, the reason for amplification failure seems not to be the low copy number of the gene.

Sequencing from single pollen grains opens up the possibility of minute sampling of herbarium specimens, which would make curators of herbarium collections more inclined to give permission for the study of unique herbarium samples. Pollen grains may possess an advantage over leaves for research purposes in that they better preserve DNA. Apart from the advantage related to the minute amount of tissue needed to do PCR, another advantage is that this technique eliminates the laborious DNA extraction step. However, the technique may have to be modified for use on pollen grains from species other than grasses.

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