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PAS-histidine kinases PHK1 and PHK2 exert oxygen-dependent dual and opposite effects on gametophore formation in the moss *Physcomitrella patens*

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ABSTRACT

Two-component systems, versatile signaling mechanisms based on phosphate transfer between component proteins, must have played important roles in adaptation and diversification processes in land plant evolution. We previously demonstrated that two Per-Arnt-Sim (PAS)-histidine kinases, PHK1 and PHK2, repress gametophore formation in the moss *Physcomitrella patens* under aerobic conditions, and that, in eukaryotes, the presence of their homologs is restricted to early-diverging streptophyte linages. We assessed here whether or not PHKs play a role in oxygen signaling. When submerged under water, the double disruption line for *PHK1* and *PHK2* formed fewer gametophores than the wild-type line (WT) both under light-dark cycles or continuous light, indicating that PHKs promote gametophore formation under an aquatic environment, in contrast to aerobic conditions. Similarly, in an artificial low-oxygen condition, the double disruption line formed fewer gametophores than WT. These results indicate that PHKs exert dual and opposite effects on gametophore formation depending on oxygen status. This study adds important insight into functional versatility and evolutionary significance of two-component systems in land plants.

1. Introduction

Upon colonization of land from aquatic environments, land plants faced a number of novel and tough environmental challenges, such as desiccation, a greater influence of gravity and stronger UV irradiation [1,2] and they have diverged and become adapted to a variety of land environments, finally filling many different ecological niches [2–4]. In these processes of adaptation and diversification, two-component systems (TCSs) have probably played important roles. Except for animals, TCSs constitute a major class of signaling pathways broadly observed in prokaryotic and eukaryotic organisms [5]. Land plants generally use “multistep” TCSs, in which (1) a “hybrid” histidine kinase (HK) autophosphorylates upon perception of a stimulus and this signal is transduced by transferring the phosphate to its receiver domain; (2) the phosphate is relayed to a histidine-containing phosphotransmitter (HPt); (3) the HPt relays the phosphate to a downstream response regulator (RR); (4) the RR transduces the signal through its output domain to modify transcription of downstream genes [6]. TCSs function as versatile signaling circuitries for responding to different biotic and abiotic environmental stimuli that are received by various sensor domains attached to HK [7]. Over the course of evolution of land plants, TCSs are also supposed to have evolved, probably by acquisition and/or loss of their components, which would have been accompanied by rewiring of their signaling circuitries. A comparison of TCSs between diverse plant lineages could help to shed light on how TCSs have changed and contributed to the evolution of plants. Of particular interest are TCSs of basal land plants, which are descendants of very early diverging lineages of

Abbreviations: TCS, Two-component system; HK, Histidine kinase; PAS, Per-Arnt-Sim; LL, Continuous light.

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embryophytes [1].

Physcomitrella patens is a species of Bryopsida, which diverged from the lineages leading to vascular plants at least 450 million years ago [8,9]. *P. patens* has been established as a model plant because various molecular biology techniques can be applied to it [8]. These features have made *P. patens* a model species of choice for studying growth, physiology and development in terms of plant evolution and diversity [8,10]. *P. patens* has two paralogous hybrid HK genes, *PHK1* (for *PAS-HK1*) and *PHK2* (*PAS-HK2*), both of which possess two Per-Arnt-Sim (PAS) domains in their N-termini [11,12]. PAS domains are found throughout the kingdoms of life, and they function as sensing units for a diverse array of signals, including molecular oxygen, small metabolites and light [13]. Thus, PAS domains are highly important for environmental adaptation. In plants, various proteins that contain a PAS domain(s) are known to regulate responses to distinct environmental stimuli [13], however, few functional analyses of PAS-containing HKs have been performed to date. In a previous report [12], we demonstrated that: i) earlier gametophore formation occurred, and ii) induction of caulonema branching by red light was up-regulated in disruption lines for *PHK1* and/or *PHK2* in *P. patens*. These observations indicate that PHK1 and PHK2 suppress the induction of caulonema branching by light, thereby delaying gametophore formation, because gametophores are developed from a portion of side branch initial cells of caulonemata [8,10]. Interestingly, in land plants, homologs of *PHK* genes are only found in relatively early-diverging lineages, *i.e.*, bryophytes and lycophytes [12]. While these plants live on land, they still depend heavily upon an aqueous environment for their reproduction, therefore we thought that PHK1 and PHK2 might be involved in the alterations between aquatic and aerial environments.

In this research, we grew *PHK1* and *PHK2* disruption lines under a state of water submergence to investigate whether PHKs have any function in aquatic environments. The double disruption line formed fewer gametophores than WT when submerged, unlike in the aerobic condition where the number of gametophores increased in disruption lines. Moreover, under the hypoxic condition as well, the double disruption line formed fewer gametophores than WT, consistent with the idea that oxygen sensing is involved in the mechanism for judging whether or not an environment is aqueous. The functional significance of PHKs in the life cycle of moss (and other relatively early-diverging plants) is discussed.

2. Materials and methods

2.1. Plant materials and growth conditions

Physcomitrella patens ssp. patens [14] was maintained in continuous light irradiated from white fluorescent lamps (light intensity: ~45 μmol m^{-2} sec^{-1}) at 25 °C unless otherwise stated. The *PHK1* single disruption line (*phk1*-13), the *PHK2* single disruption line (*phk2*-44) and the *PHK1/PHK2* double disruption line (*phk1 phk2*-20) were generated in our previous report [12]. *P. patens* protonemata were grown on BCDAT medium [15], collected every three to seven days and ground with a homogenizer (Physcotron, Microtec, Funabashi, Japan), then applied to a new BCDAT agar plate [15].

2.2. Low oxygen conditions

For the water-submerged condition, small tissue colonies (~1 mm in diameter), taken from protonemata grown on BCDAT medium for three days in continuous light, were inoculated and grown for one week in continuous light (LL) or in a light-dark cycle (8 h-light/16 h-dark) on BCDAT medium. These colonies were then submerged in water and grown in the same light conditions for three weeks. To submerge colonies, ~30-ml sterilized water was poured into a plastic plate with a diameter of 10 cm, in which tissue colonies were grown on ~25-ml of solidified BCDAT agar medium. For the hypoxic condition, small tissue colonies that had been inoculated on BCDAT medium were incubated for three weeks in LL or in light-dark cycles in an airtight 2.5-l jar (Mitsubishi Gas Chemical Company, Tokyo, Japan), from which oxygen was absorbed by an absorbent (AnaeroPack for hypoxic culture; Mitsubishi Gas Chemical Company). Final oxygen concentration was 6–12%. Resulting colonies were observed under a stereomicroscope (SZX16, Olympus, Tokyo, Japan) and their images were taken with a digital camera (DP21, Olympus).

3. Results

To study whether *P. patens* PHKs play a role in an aquatic environment, we grew protonema tissue colonies of the disruption lines for *PHK1* and/or *PHK2* (*PHK1* single disruption line, *phk1*-13; *PHK2* single disruption line, *phk2*-44; *PHK1 PHK2* double disruption line, *phk1 phk2*-20), which we established in a previous report [12], along with WT for a week. We then i) kept them in the same condition as the control (aerobic condition) or ii) transferred and grew them in water (submerged condition), in LL or in light-dark cycles for three weeks. We used a short-day condition (8 h-light/16 h-dark cycles) for light-dark cycles, because phenotypic differences between WT and the disruption lines under aerobic condition were more conspicuous in light-dark cycles with this day/night length than in the cycles with other day/night lengths [12]. Under the aerobic condition, while there were no significant differences between WT and the disruption lines in LL (Fig. 1 (B), open bars), all the disruption lines formed significantly more gametophores than WT in light-dark cycles (Fig. 1 (C), open bars). These observations are consistent with our previous report, in which the disruption lines formed more gametophores than WT unless they were grown for a period longer than a certain cumulative length of light (*i.e.*, grown under LL or the long-day condition for four weeks), in which case no differences were observed between WT and the disruption lines [12]. These results (from this study and the previous report [12]) indicate that PHK1 and PHK2 suppress gametophore formation thereby delaying its timing but do not affect the final number of gametophores. On the other hand, we obtained contrasting results when the protonema colonies were grown under submergence. In LL, the double disruption line formed fewer gametophores than WT and single disruption lines (22.2, 18.4, 20.4 and 11.8 for WT, *phk1*-13, *phk2*-44 and *phk1 phk2*-20, respectively; Fig. 1 (B), close bars). In light-dark cycles, the single disruption lines as well as the double disruption line formed fewer gametophores than WT (2.6, 0.6, 1.0 and 0.6 for WT, *phk1*-13, *phk2*-44 and *phk1 phk2*-20, respectively; Fig. 1 (C), closed bars). These results indicate that PHK1 and PHK2 promote gametophore formation when submerged, and hence, they have dual and opposite regulatory effects on gametophore formation depending on whether the protonema cells are grown aerobically or submerged in water. In addition, when submerged, protonema filaments of the double disruption line were sparser than those of WT and single disruption lines in LL (Fig. 1 (A), upper photos). In contrast, all the lines showed sparse filaments and no significant difference was observed in protonema density between the lines in light-dark cycles (Fig. 1 (A), lower photos).

Oxygen is strictly limited to cultured cells that are submerged. Therefore, we grew WT and disruption lines under an artificial hypoxic condition (concentration of O_2: 6–12%) for three weeks to compare the number of gametophores between lines, in order to investigate whether oxygen availability is a factor that determines

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