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Seasonal changes in ice nucleation activity in blueberry stems and effects of cold treatments in vitro

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ABSTRACT

Ice nucleation activity (INA) of plant intrinsic origins is considered to play important roles in plant cold hardiness mechanisms. Yet, only a few studies have addressed the spatial and temporal localization of plant INA, how it is regulated and what its functional roles are. In our previous study (Kishimoto et al., 2014), we revised a test tube method and developed a highly reproducible assay for measuring INA of plant specimens and demonstrated that high INA occurred in the cell wall fraction of wintering bark tissues of blueberry stems and corresponded well to the freezing behavior (extracellular freezing) of the stem bark. Here, we followed precisely seasonal changes in the stem INA of two blueberry cultivars and alterations in the stem INA caused by artificial incubation at various low temperatures. INA of newly developed shoots was low but increased rapidly by July when the stem became seemingly matured, then gradually increased with the maximum in October or early November just before the first autumnal frost. Following the subsequent recurrent frosts, the stem INA gradually decreased. This tendency was consistent between the two cultivars differing in the level of cold hardiness. INA in the stems of September until February was increased by incubation at 0-7 °C whilst decreased by freezing to lower temperatures. The *in vitro* results corroborate the seasonal changes in the stem INA in the field but the mechanisms remain to be investigated. The highest level of INA (expressed as the median ice nucleation temperature) observed with current year stems (7.5 mm-long) of Woodard in October of 2010-2013 was -0.9 ~ -1.0 °C when determined with 2 mL assay system (-1.1 ~ -1.3 °C with 0.5 mL system). This may likely be one of the highest INA of biological origins ever reported.

1. Introduction

Regulation of freezing in plant tissues is a key issue in cold hardiness mechanisms but still remains obscure (Ishikawa et al., 2009). This includes regulation of ice nucleation and propagation, inhibition of ice growth (e.g., antifreeze, recrystallization inhibition and morphological barriers), control of water flow, stabilization of supercooling and control of thawing. Among them, ice nucleation or how the plant initiates freezing is the primary event when the plants encounter subfreezing temperatures and is a vital part of freeze regulation (Pearce, 2001; Wisniewski et al., 2009).

Theoretically, ice nucleation of plants can be caused by extrinsic ice such as snow, frosts, frozen soil or already frozen parts of the plant, also by epiphytic ice-nucleating bacteria and by plant intrinsic ice nucleation activity (the ability of tissues to cause ice nucleation, hereafter referred to as INA). The functional roles and contribution of plant intrinsic INA in ice nucleation and cold hardiness of wintering plants remain unanswered. It may be involved in initiating spontaneous freezing of the whole plant at high subzero temperatures under dry surface conditions in the absence of external ice (Kishimoto et al., 2014). Plant intrinsic INA may also contribute to the ice nucleation at specific sites to properly initiate and regulate tissue- and species-specific freezing behaviors of cold hardy plant tissues such as extracellular freezing and extra-organ freezing (Ishikawa and Sakai, 1985; Ishikawa et al., 2009). It may also be involved in the accumulation and retention of ice crystals in appropriate and specific sites in the tissues. More studies are required to characterize plant intrinsic INA on precise spatial/temporal localization and distributions in the plant

Abbreviations: INA, ice nucleation activity; INT, ice nucleation temperature.

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kingdom and how it is regulated and evolved. Previous attempts to identify plant intrinsic ice nucleators ended up with only partial characterization (e.g., Ashworth and Davis, 1984; Gross et al., 1988; Brush et al., 1994) and the substances responsible for the INA have not been successfully identified (Wisniewski et al., 2009). Accumulation of more knowledge on this physiologically important trait as a cold hardiness mechanism and development of an accurate and reproducible assay system to realize such analyses seem essential.

In our previous study (Kishimoto et al., 2014), we revised the test tube nucleation assay (Ashworth and Davis, 1984; Gross et al., 1988; Hirano et al., 1985) and developed a highly reproducible assay for determining INA of plant specimens. Our assay is characterized by the use of smaller systems (0.5-2 mL of water in a tube) with smaller samples and the elimination of possible foreign ice nucleators by autoclaving and clean handling procedures though it uses a similar apparatus (Fig. 1). Using this assay, we surveyed INA of various tissues of more than 600 species (e.g., Ishikawa, 2014; Sekozawa et al., 2002; Ueda et al., 2002) and found an extremely high INA in blueberry cultivars. This INA was associated with stems, where it was localized in the cell wall fraction of bark tissues (Kishimoto et al., 2014). The stem INA was resistant to various antimicrobial treatments and showed highly consistent and specific localization in the tissues, which implies that the stem INA was most likely of intrinsic origin rather than microbial origin (Kishimoto et al., 2014). The localization of INA corresponded well to the freezing behavior (extracellular freezing) of the bark, ice accumulation in the bark and initiation of freezing in the stem. The high INA in blueberry stems seems to contribute to avoiding excess supercooling and spontaneously initiating freezing in the extracellular space of the bark by acting like a subfreezing temperature sensor. To further clarify the functional roles and identity of this high stem INA, studies are required to reveal when and how the INA of blueberry stems develops and changes seasonally in relation to growth/development, cold temperatures and cold hardiness/cold acclimation.

Seasonal alterations in INA have been studied with regard to the role of epiphytic ice nucleating bacteria in frost injuries of crops and fruit trees. Population dynamics of ice nucleating bacteria and their INA were extensively followed (Gross et al., 1983; Hirano et al., 1985; Rajashekar et al., 1983; Gusta and O'connor, 1987). Yet, the contribution of microbial INA seemed minor in wintering perennial tissues (Ashworth and Kieft, 1995). Only a few studies have shown chronological changes in the INA of plant intrinsic origins (Ashworth et al., 1985a; Gross et al., 1988; Sekozawa et al., 2002).

The objective of the present study is to further characterize the INA in blueberry stems, especially to clarify how the INA develops and changes seasonally in the field, what environmental factors (cold or freezing) affect the INA, how it is induced or decreased *in vitro* and to see if there are any differences between high bush and rabbit eye blueberries and between current and previous year stems. It was also intended to demonstrate the highest levels of INA that blueberry stems can acquire in field conditions and the usefulness of our revised test tube assay for determining the INA of small plant samples.

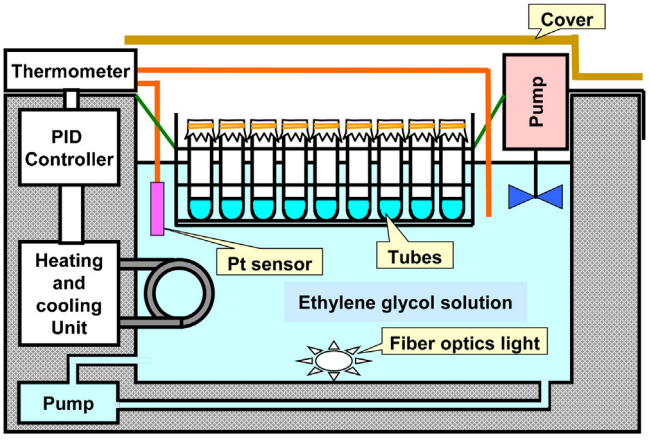

Fig. 1. A schematic illustration of the apparatus for determining INA of plant tissues using test tubes. The cooling bath was equipped with two circulating pumps and a PID (proportional-integral-derivative) controller with a Pt sensor. This allowed temperature overshooting and space- and time-wise errors of the sample temperature in the tube to be minimized less than 0.1 °C.

2. Materials and methods

2.1. Plant materials

To follow seasonal changes in the INA, we used current and previous year (1 year and 2 year-old) stems of 20 year or more-old trees (about 4 m tall) of high-bush (*Vaccinium corymbosum* L. cv. Weymouth) and rabbit-eye (*Vaccinium ashei* Reade cv. Woodard) blueberry grown in the nursery field of the University of Tsukuba, Ibaraki, Japan (approx. 50 km northeast of Tokyo). The stem samples were randomly collected in the first week of every month from May 2001 until April 2002. These two cultivars were selected to represent the two major blueberry species grown in this area with different levels of cold hardiness (approximate LT_{50} for Weymouth and Woodard in mid-winter: -25 °C and -16 ~ -18 °C, respectively). Cultivation zones in Japan for cold hardy high-bush and less cold hardy rabbit-eye blueberry cultivars are roughly from N33° to N43° and from N30° to N36°, respectively. In Tsukuba (N36° 06′), both species are routinely cultivated. The collected samples were cooled on ice and used immediately for experiments unless otherwise noted. To unravel more precise changes in the stem INA as affected by late autumnal chilling temperatures and frosts, current year stems of both cultivars were sampled every two days between November 7 and December 8 in 2001 and used for INA determination. To check the reproducibility and to identify when and how high levels of stem INA were achieved, the stem INA was also followed from autumn to winter in 2003-2013 by using 10 year or more-old blueberry trees (about 1-2 m tall) of both cultivars grown in the field of our institute (NIAS, Tsukuba).

2.2. Cold treatment of the stems in vitro

Stem samples collected in various months were also used for artificial incubation at various low temperatures to know how the stem INA was affected. Stems of both cultivars collected from September to February were put in polyethylene bags and incubated at 7 or–4 °C for 3, 6, 15, 30, 60 days and the INA of current year (1 year-old) stems was determined. Similarly, stems collected in September and October were incubated at 4 different temperatures: 7, 4, 0 and -4 °C for up to 2 months prior to determination of stem INA.

To unravel the effect of freezing temperatures on the stem INA and viability, stems collected between September and January were slowly cooled using a programmable freezer. More precisely, blueberry stems (15 cm long) enclosed in polyethylene bags, following ice-inoculation at -2 °C and equilibration at -3 °C for 6 h, were cooled (cooling rate: 2 °C/h to -12 °C, then 5 °C/h to -40 °C) to the designated temperatures (-3, -9, -12, -15, -21, -27, -40 °C). Following being rewarmed at 4 °C overnight, the current year (1 year-old) stems were immediately used for INA determination without washing and for viability determination.

2.3. Determination of ice nucleation activity (INA) of blueberry stem samples

INA of blueberry stems (1 or 2 year-old) were determined as detailed previously (Kishimoto et al., 2014) using a revised test tube

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