农业低温灾害研究新进展

李茂松 王道龙 吉田 久(日) 主编

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编　委　会

序

低温灾害，包括冻害、霜冻害、冷害等是影响中、日两国农产品产量和质量安全的重要气象灾害之一。近年来各种低温灾害的频率有不断增加的趋势。在全球气候缓慢逐渐变暖的同时，极端气候变化频率增加，冷热不均，是造成低温灾害频率增加的客观原因。同时，随着我国国民经济和社会的发展，农业生产中农作物和品种结构布局不断改变，一些特种作物的栽种范围扩大，使其受霜冻害的风险加大，也是我国低温灾害的发生变得日益频繁的原因之一。目前，低温灾害仍然是中、日两国农业生产中的重大问题。研究各种低温灾害的发生发展规律、不同作物的受害模式，提高对低温灾害的防御水平，对增强对低温灾害的抵抗能力，保障我国粮食生产和其他农产品的质量安全具有重要意义。为此，在中、日农业技术合作项目支持下，中、日双方围绕低温灾害开展了合作研究，特别是在小麦霜冻害方面，中、日双方从不同的角度开展了一系列合作研究工作。为了增进交流和相互了解，于2006年5月11～12日在北京召开“中、日低温灾害及其防御对策研讨会”。此次会议有来自双方的代表50名，就不同地区、不同作物的冻害、霜冻害、冷害等几个议题开展了讨论。

本书的出版得到了日本农业协力机构JICA的支持。希望本书的出版能够给相关研究人员对本领域的研究提供参考。由于时间有限，书中的错漏之处在所难免，恳请广大读者批评指正。

《农业低温灾害研究新进展》编委会

二〇〇六年十月

目　录

Genetic and Genomic Studies for Winter Hardiness in Grasses

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Abstract

Winter hardiness is the outcome of a number of interacting factors that include vernalization requirement, photoperiod response, low temperatures tolerance and resistance to snow moulds. An understanding of the genetic basis of these component traits permits more efficient selection based on closely linked molecular marker loci. The most consistently identified region, on homoeologous group 5 chromosomes, contain quantitative trait loci (QTL) for vernalization response, low temperatures tolerance and photoperiod sensitivity. Close genetic linkage between the major genes influencing winter hardiness and genes regulating cold-induced sugar production was observed in wheat. Recent molecular genomic approach revealed that C-repeat binding factor (CBF) genes are candidate genes for low-temperature tolerance QTL in wheat and barley. Perennial forage grasses must survive the periods of protracted snow cover and low temperature during the winter in the northern biosphere such as Hokkaido in Japan. Perennial ryegrass (*Lolium perenne* L.) and orchardgrass (*Dactylis glomerata* L.), however, is susceptible to winter stresses caused by both snow moulds and low temperatures. Thus winter hardiness is a very important character in breeding program of these species. Substantial advances have been made in the genetic improvement of plant populations through artificial selection of winter hardiness. Most of this selection has been on the basis of observable phenotype, without knowledge of the genetic architecture of winter hardiness. Thus the development of high-resolution genetic maps would make it possible to identify the chromosomal regions, or in some instances, the individual sequence variants that are responsible for trait variation. Some QTLs for traits involving winter hardiness have been identified in forage grasses. Some candidate genes for winter hardiness such as sugar metabolism, glycine-rich RNA binding protein and CBF have been analyzed. Comparative genomic analysis revealed that conserved synteny with winter hardiness has been observed between Triticeae and forage grasses.

Winter hardiness

To survive the winter, a plant must evolve mechanisms whereby freezing-sensitive tissues can avoid freezing or undergo freezing tolerance compatible with the normal variations of the local climate, co-ordinate the induction of the tolerance at the appropriate time, maintain adequate tolerance during time of risk, and properly time the loss of tolerance and resumption of growth when the risk of freezing has passed (Guy, 1990). Winter-hardiness is a complex trait (Fig. 1), factors involved including a) freezing temperatures, b) fluctuating temperatures, c) wind, d) snow cover, e) ice encasement, f) heaving, g) low-light and h) low-temperature pathogens.

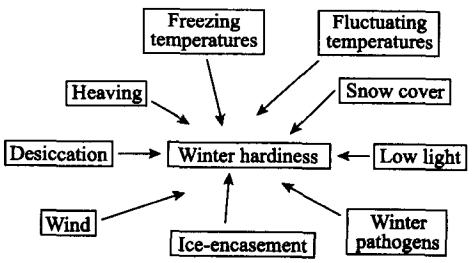


Fig. 1 Winter hardiness is a complex trait

Development of winter-hardiness requires exposure of plants to low non-freezing temperatures typically 0℃ to 10℃, and shortened photoperiod. Many physiological and biochemical changes occur during cold acclimation, including slowed or arrested growth, reduced tissue water content, altered cell pH, protoplasm viscosity and photosynthetic pigments, reduced ATP levels (Levitt, 1980), transient increases in ABA (Chen et al., 1983), changes in membrane lipids (Uemura and Steponkus, 1994), accumulation of compatible solutes including proline, betaine, polyols and soluble sugars, and accumulation of antioxidants (Tao et al., 1998).

The rate and extent of de-hardening is a critical factor in winter survival. Overwintering plants are particularly susceptible to freezing damage in the spring if the deacclimation process occurs prematurely or too rapidly, or if unpredictable temperature fluctuations occur (Levitt 1980; Gay and Eagles 1991). Recently global warming may influence unpredictable temperature fluctuations. Eagles (1989) suggested that the nature of an adaptive cold acclimation process would vary with the stability and predictability of winter conditions in a particular environment. In stable and predictable cold continental climates where the onset of freezing temperatures is rapid, a photoperiod-triggered and rapid acclimation process is desirable, while in the more variable and less severe conditions of maritime climate, a temperature dependent response might enable plants to exploit a mild autumn or spring by continuing to grow. However, in cultivars adapted to maritime climates, deacclimation may occur in response to fluctuations in winter and spring temperature with a risk of damage by subsequent frosts (Eagles, 1994).

Molecular mechanisms involved in cold acclimation are largely unknown but information from model species whose genomes have recently been sequenced such as *Arabidopsis* and rice and the development of microarray technologies are giving insight into the complexity of the processes (Seki et al., 2001; Shinozaki et al., 2003; Shinozaki and Yamaguchi-Shinozaki, 2006). The CBF (C-repeat binding factor) /DREB1 (dehydration-responsive element-binding protein 1) regulon is the most important transcription unit involved in cold acclimation in plants (Nakashima and Yamaguchi-Shinozaki 2006).

Genetic analysis of winter hardiness

Triticeae

Winter hardiness is the outcome of a number of interacting factors that may include vernalization requirement, photoperiod response, low-temperature tolerance and resistance to snow moulds. An understanding of the genetic basis of these component traits permits more efficient selection based on closely linked molecular marker loci. In the Triticeae cereals, quantitative trait loci (QTL) analysis has identified a limited number of conserved genome regions as responsible for the winter hardiness character. The most consistently identified region, on homoeologous group 5 chromosomes, contains QTLs for vernalization response, low temperature tolerance and photoperiod sensitivity (Pan et al. 1994; Cattivelli et al. 2002). These QTL effects have been described as the effects of single loci. Low-temperature tolerance loci on chromosomes 5A, 5B, and 5D of wheat have been given the locus designation *Fr-A1*, *Fr-B1* and *Fr-D1*, respectively (Sutka and Snape, 1989; Snape et al., 1997; Toth et al., 2003). The vernalization loci have been assigned a *Vrn* prefix, and orthologues of the *T. monococcum Vrn1* locus map to the homoeologous 5 chromosomes (Cattivelli et al., 2002). The *Vrn* gene nomenclature was standardized and map locations were further refined by Dubcovsky et al. (1998). RFLP analysis demonstrated that vernalization requirement and frost resistance are controlled by two different, but tightly linked loci (*Vrn-A1* and *Fr-A1*) on chromosome 5A of wheat (Galiba et al., 1995; Sutka et al., 1999). Because of the observed large effect on frost resistance, molecular marker-assisted selection for the *Vrn-A1-Fr-A1* chromosomal region has been proposed as a method for improvement of cold hardiness in wheat cultivars (Storlie et al., 1998). Wheat near isogenic lines

with different vernalization alleles have been evaluated for cold hardiness in order to assess the viability of this strategy (Koemel et al., 2004). Close genetic linkage between the major genes influencing winter hardiness and genes regulating cold-induced sugar production was also observed in wheat (Galiba et al., 1997). In addition, QTLs controlling traits associated with 'winter' hardiness, such as field survival and crown fructan content, were mapped to the long arm of chromosome 5H in a cross between winter 'x spring' barley varieties (Hayes et al., 1993; Pan et al., 1994). Recently, comprehensive measurements of low temperature tolerance and vernalization requirement were used for analysis of a new winter 'x spring' barley population, and a QTL for accumulation of proteins encoded by COR (COld Regulated) genes on chromosome 5H (*Cor14b*, *tmc-ap3*) was coincident with a QTL for low temperature tolerance (Francia et al., 2004). CBF family genes have been shown to be key determinants of low temperature tolerance (Thomashow, 1999; Thomashow et al., 2001). CBF gene family was mapped in a QTL for low temperature tolerance in barley (Francia et al., 2004; Tondelli et al., 2006). In wheat, CBF3 was also linked to the frost-tolerance locus *Fr-A2* on chromosome 5A (Vágújfalvi et al., 2003).

Forage grasses

Evaluation and breeding of winter hardiness

Perennial forage grasses must survive the periods of protracted snow cover and low temperature during the winter in the northern biosphere such as Hokkaido in Japan. Perennial ryegrass (*Lolium perenne* L.) and orchardgrass (*Dactylis glomerata* L.), however, is susceptible to winter stresses caused by both snow moulds and low temperatures. Thus winter hardiness is a very important character in breeding program of these species in Hokkaido region, Japan.

Freezing tolerance is usually expressed as LT_{50} (the median lethal temperature that kills 50% of plants) by cooling of crown tissues from hardened plants using a programmed freezer. Table 1 shows the LT_{50} of some grasses and cereals. Kentucky bluegrass and bent grass are extremely hardy and perennial ryegrass is susceptible for freezing stress.

Table 1 Freezing tolerance in grasses

Tolerance	LT_{50} (℃)	Grasses
Extremely high	−30 ~ −40	Kentucky bluegrass, bentgrass
High	−25 ~ −30	Red fescue, meadow fescue, timothy, rye
Medium	−20 ~ −25	Orchardgrass, wheat
Relatively weak	−15 ~ −20	Perennial ryegrass, barley

LT_{50} is changed in seasons. LT_{50} decreases during autumn and early winter, and the lowest value is observed at December and January. Then LT_{50} increases to spring. Fig. 2 shows the seasonal changes of LT_{50} of three representative forage grasses in Hokkaido region.

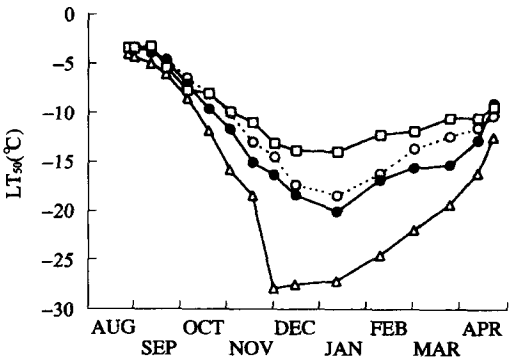


Fig. 2 Seasonal change of LT_{50} in grasses

Evaluation test for genetic resources of ochardgrass including 21 European and four Japanese cultivars revealed that there was a significant correlation between freezing tolerance and resistance to snow mold as *Typhula ishikariensis* ($r = 0.89$, $p < 0.001$, Fig. 3) and a significant negative correlation between freezing tolerance and autumn vigor ($r = -0.72$, $p < 0.01$, Fig. 4) (Nakayama et al. 1997).

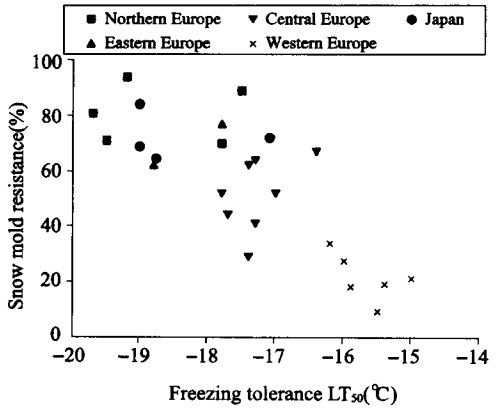


Fig. 3 Relationship between freezing tolerance (LT_{50}) and resistance to snow mold by *T. ishikariensis* in*Dactylis glomerata* populations (Nakayama et al. 1997)

Breeding program in orchardgrass and meadow fescue in the National Agricultural Research Center for Hokkaido Region (NARCH) has been carried out using germplasm introduced from Russia, whose collections had high freezing tolerance and snow mold resistance. However, many Russian germplasm showed autumn dormancy with less productivity in autumn. Then superior lines with good winter hardiness and high yield have been selected from the crosses between Japanese cultivars and Russian germplasm at NARCH (Yamada et al. 2006).

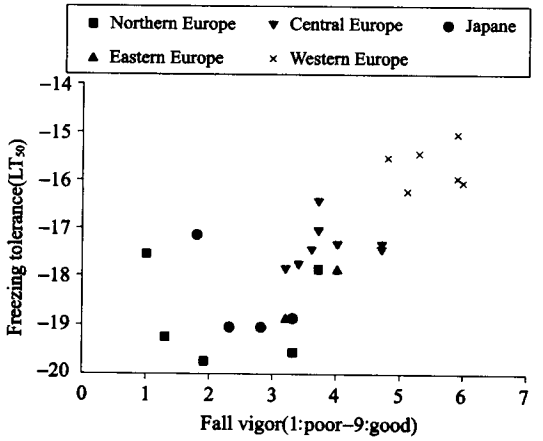


Fig. 4 Relationship between freezing tolerance (LT_{50}) and fall vigor in *Dactylis glomerata* populations (Nakayama et al., 1997)

Linkage analysis

An enhanced molecular marker – based genetic linkage map of perennial ryegrass has recently been constructed through the activities of the International *Lolium* Genome Initiative (ILGI) (Forster et al., 2001), using the p150/112 one – way pseudo – testcross mapping population. The current map contains 109 RFLP loci detected by heterologous probes from wheat, barley, oat and rice. Comparative genetic mapping has allowed the alignment of the perennial ryegrass genetic map with those of wheat, rice and oat, revealing substantial conserved synteny with the genomes of Poaceae species (Jones et al., 2002). The syntenic chromosomal regions are represented as a concentric circle alignment (Fig. 5). At the macrosyntenic level, each of the 7 linkage groups of perennial ryegrass chiefly corresponds to one of the seven basic homeologous chromosome groups of the Triticeae cereals, and they have been numbered accordingly. Seven linkage groups of perennial ryegrass also correspond to 12 linkage groups of rice.

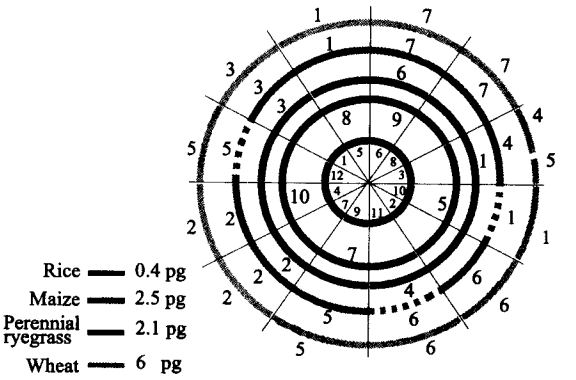


Fig. 5 Concentric circle alignment of Poaceae genome (from Jones et al., 2002)

No significant QTLs for winter survival in the field were identified in the reference map of p150/112 perennial ryegrass (Yamada et al., 2004). However, a QTL for electrical conductivity corresponding to frost tolerance (Dexter et al., 1932) was located close to a heading date QTL in a region is likely to show conserved synteny with chromosomal regions associated with both winter hardiness and flowering time variation in cereals (Yamada et al.,

2004).

The F_2 (Aurora × Perma) genetic map population (Armstead et al. 2002, 2004) was used to identify QTLs for traits relating to winter hardiness, as well as sugar content. Single genotypes from partially (third generation) inbred lines derived from two agronomically contrasting varieties (cultivars Aurora and Perma) were pair – crossed at IGER, UK. A single F_1 hybrid genotype was self – polinated to derive an F_2population of 180 genotypes. The F_2 (Aurora × Perma) genetic map population was evaluated for freezing tolerance with crown tissues from natural hardened genotypes using a programmed freezer. Freezing tolerance in tillers was also evaluated and QTL for this trait was observed on linkage group (LG)s 5 and 6. Then the map population was evaluated for winter hardiness in the field condition at Sapporo. Snow mould – resistant varieties accumulate higher levels of fructan and metabolize them at slower rates compared to susceptible varieties (Yoshida et al., 1998). Many of the snow mould fungi, such as *Typhula* spp., *Microdochium nivale* and *Myriosclerotinia borealis*, can co – infect on single plants, and their interactions may obscure the respective effects on plant survival (Matsumoto and Araki, 1982; Matsumoto et al., 1982). The use of fungicides with a limited spectrum of activity may clarify these specific effects. *Typhula* snow moulds such as *T. ishikariensis* and *T. incarnata* generally occur in the deep snow environment of the western region of Hokkaido, Japan, including Sapporo. In this environment, control of *S. borealis* and *M. nivale* infections with the fungicide iminoctadine – triacetate is an effective method for evaluation of resistance to *Typhula* snow moulds (Takai et al., 2004). Scores of winter survival were measured in the F_2 (Aurora × Perma) population using this control regime, and QTLs for this trait were identified on LGs 2, 4, 6 and 7. Fructan content was also measured by high performance liquid chromatography (HPLC) using crown tissues from plants grown outdoor in December. QTLs for content of high molecular fructan with more than eight degrees of polymerization (DP) were observed on LGs 1, 2 and 4. QTLs for winter survival in LGs 2 and 4 are close to QTLs for high molecular weight fructan content.

Genomic studies in grasses

Genetic transformation

The accumulation of fructan, a polymer of fructose and a major component of nonstructural carbohydrates, in grasses during autumn is linked to winter hardiness. Genetic manipulation of the accumulation of fructan could be an important molecular breeding strategy for the improvement of winter hardiness in grasses. Transgenic perennial ryegrass plants that over – expressed the wheat fructosyltransferase genes, *wft1* and *wft2*, which encode sucrose – fructan 6 – fructosyltransferase (6 – SFT) and sucrose – sucrose 1 – fructosyltransferase (1 – SST), respectively, under the control of CaMV 35S promoter have been produced using a biolistic transformation (Hisano et al. 2004). Transgenic plants that accumulated a greater amount of fructan than non – transgenic plants showed increased tolerance to cellular freezing. The results suggest that the over – expression of the genes involved in fructan synthesis serves as a novel strategy to produce freezing – tolerant grasses (Hisano et al. 2004).

Candidate gene approach

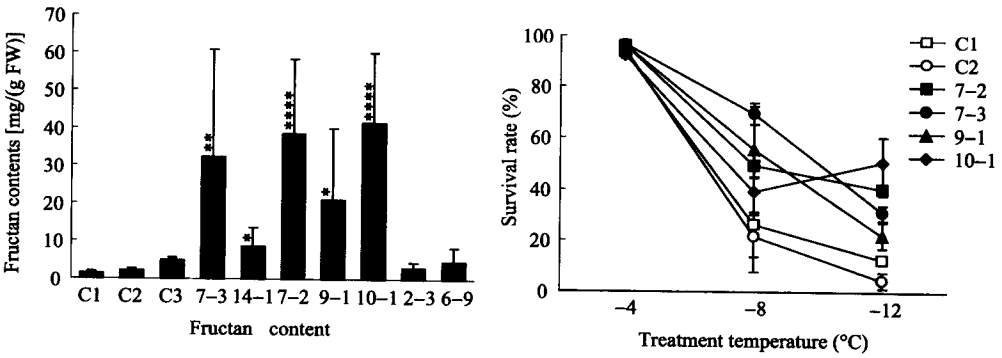
The next generation of molecular genetic markers for forage grasses will be derived from expressed sequences, with an emphasis on functionally – defined genes associated with biochemical and physiological processes that are likely to be correlated with target phenotypic traits (Forster et al., 2004; Faville et al., 2005). Candidate gene approach would be promising as perfect DNA markers for marker – assist breeding selection.

The α – subunit of the casein protein kinase CK2 has been implicated in both light – regulated and circadian rhythm – controlled plant gene expression, including control of flowering time. Two putative CK2 α genes of perennial ryegrass (*Lpck2a* – 1

and *Lpck2a* – 2) have been obtained from a cDNA library constructed with mRNA isolated from cold-acclimated crown tissue (Shinozuka et al., 2005). The *Lpck2a* – 1 CAPS marker was assigned to perennial ryegrass LG 4 and the *Lpck2a* – 2 CAPS marker was assigned to LG2. Allelic variation at the *Lpck2a* – 1 and *Lpck2a* – 2 gene loci was correlated with phenotypic variation for heading date and winter survival, respectively (Shinozuka et al., 2005).

The gene for a putative glycine – rich RNA binding protein, *LpGRP*1, was isolated from a cDNA library constructed from crown tissues of cold-treated perennial ryegrass plants (Shinozuka et al., 2006). An RFLP locus detected by the *LpGRP*1 cDNA probe was mapped to a distal location on LG 2 in the p150/112 population. A full length sucrose synthase cDNA (*LpSS*) from perennial ryegrass was isolated and CAPS marker for this was mapped on LG 7 in the F_2 (Aurora × Perma) population (Bhowmik et al., in preparation).

Genes of fructan biosynthesis enzyme, fructosyltransferase as 1 – SST, 1 – FFT and 6G – FFT were isolated from perennial ryegrass and characterized by heterologous expression in the *Pichia pastoris* system (Hisano et al., in preparation). *Lp*1 – *SST* and *Lp*1 – *FFT* mapped to the upper region of LG 7 in the F_2 (Aurora × Perma) genetic map, but failed to show coincidence with any fructan content QTLs. The *Lp*1 – *SST* gene (Chalmers et al., 2003) was also assigned to the equivalent region of LG 7 as a single nucleotide polymorphism (SNP) locus in the F_1 (NA_6 × AU_6) second – generation reference family (Faville et al., 2004). However, *Lp6G* – *FFT* mapped to LG 3 close to a QTL for low – molecular weight fructan content (Hisano et al., in preparation).



C1, C2: non transgenic plants regenerated from calli
C3: a plant grown from a seed.
7 – 3, 14 – 1: trangenic with wheat 6 SFT gene
7 – 2, 9 – 1. 10 – 1: transgenic with wheat 1 SST gene
2 – 3, 6 – 9: transgenic with both genes.

Fig. 6 Transgenic perennial ryegrass plants overexpressed with wheat 6 SFT and 1 SST genes under control of CaMV 35S promoter using a particle bombardment method accumulated increased of fructan and increased freezing tolerance (Hisano et al. 2004).

The CBF/DREB1 genes are the most important regulators in the cold signaling pathway in many plants. CBF regulons were reported to be candidate genes for low-temperature tolerance QTL in wheat and barley (Skinner et al., 2006). 10 novel putative CBF cDNAs of perennial ryegrass have been isolated from cold-treated leaf tissue (Tamura et al., in submission). Their primary structures contain some conserved motifs characteristic to CBF gene family. Phylogenetic analysis revealed that *LpCBF* genes were classified into the HvCBF3 – , and HvCBF4 – subgroups following the previously proposed classification

of barley CBF genes. RT – PCR analysis revealed that the expression of *LpCBF* genes was rapidly induced in response to low temperature and that the expression pattern under the low temperature conditions for a long period was different among the various CBF genes. We mapped five of the 10 *LpCBF* genes on a genetic linkage map using the p115/112 reference population. The *LpCBFIb*, *LpCBFII*, *LpCBFIIIb* and *LpCBFIIIc* genes were mapped on LG5 forming a cluster within 2.2cM, while *LpCBFVb* was mapped on LG1. Conserved synteny for CBF gene family was observed between the Triticeae cereals and perennial ryegrass. In the p150/112 population, a QTL for electrical conductivity corresponding to frost tolerance was located on the upper region of LG4, while QTL related to low temperature tolerance have not been found near the CBF gene cluster (Yamada et al. 2004). In the 'Aurora × Perma' F_2 genetic map population of perennial ryegrass, QTLs for freezing tolerance in tillers were observed on LGs 5 and 6. We developed in/del marker of the *LpVRN*1 gene and mapped the locus on LG4 in the p150/112 population as reported by Jensen et al. (2005).

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